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Señor Director
DIRECCION DE NUEVAS CREACIONES
Superintendencia de Industria y Comercio
E. S. D.

Trámite : 011
Evento : 379
Actuación : 444

Referencia : Expediente No. **07.080.236**
Asunto : Solicitud de Patente para: **MATRICES COMPLEMENTADAS PARA LA REPARACIÓN DE FRACTURAS ÓSEAS**
Solicitante : BAXTER INTERNATIONAL INC. AND BAXTER HEALTHCARE S.A.
Fecha solicitud : 6 de agosto de 2007

1. Yo, Luz Clemencia DE PÁEZ, en mi condición de apoderada de la sociedad solicitante de la patente de la referencia, me refiero al oficio No. **08752** notificado por fijación en lista del 30 de mayo de 2012.

2. Mediante oficio No. **08752** se puso en conocimiento del solicitante el informe que contiene las observaciones hechas por su Despacho sobre la presente solicitud de patente. Las observaciones realizadas en el oficio son las siguientes:

3.1 <u>Reivindicaciones</u>

De manera resumida, el Examinador ha expuesto lo siguiente:

- Reivindicaciones 21, 22 y 23: este uso no es patentable de acuerdo con el artículo 14 de la Decisión 486 de la Comisión de la Comunidad Andina.

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3.2 Análisis de patentabilidad

3.2.1 Nivel inventivo

El Examinador considera que la combinación de las enseñanzas de los documentos D1: WO03052091 y D2: "European Cells and Materials" afecta el nivel inventivo de la presente solicitud.

Específicamente se afirma que la persona medianamente versada en la materia incluiría una u otra concentración de un péptido PTH_{1-34} ó un péptido de fusión PTH (proteínas plasmáticas), dado que este tipo de formulación se ha utilizado durante más de dos décadas porque no produce rechazo inmunológico, según da a conocer D2, en la formulación de D1, llegando de tal manera a la formulación de la reivindicación 1 de la solicitud.

4. Respuesta a las objeciones

De este modo y con el objeto de atender las objeciones arriba mencionadas, se desea presentar las siguientes modificaciones, aclaraciones y argumentos:

4.1 Modificación de una solicitud en trámite

De acuerdo con el artículo 34 de la Decisión 486 de la Comisión de la Comunidad Andina, donde se establece que la modificación de una solicitud es posible en cualquier momento del trámite mientras no implique una ampliación de la solicitud inicial, me permito presentar un nuevo pliego reivindicatorio conformado por 20 reivindicaciones.

Así, siguiendo las amables sugerencias del Examinador, se han introducido las siguientes modificaciones al pliego reivindicatorio:

- Se eliminan las reivindicaciones 21, 22 y 23.

- La reivindicación 1 ha sido modificada incluyendo "Una formulación *adecuada para la reparación de fracturas óseas* que comprende".
- La reivindicación 13 ha sido modificada incluyendo "Una matriz complementada *adecuada para la reparación de fracturas óseas* que comprende".

Por lo anterior, las objeciones formuladas por el Examinador en cuanto a la patentabilidad de las reivindicaciones 21, 22 y 23, del pliego reivindicatorio se ven superadas. En consecuencia, se solicita atentamente tener en cuenta este nuevo pliego reivindicatorio para el análisis de patentabilidad.

4.2 Argumentos a favor del nivel inventivo de la presente solicitud

El artículo 18 de la Decisión 486 señala lo siguiente:

> **Artículo 18.-** Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica.

Consecuentemente, una invención se considerará inventiva si la misma no hubiese resultado obvia a partir del arte previo, para una persona del oficio normalmente versada en la materia. Esto a su vez implica que el Examinador a cargo de esta evaluación debe colocarse en el momento exacto en el que el inventor buscaba resolver el problema técnico contemplado en la solicitud y para el cual poseía la literatura y el conocimiento disponibles en dicho momento.

Siguiendo entonces los lineamientos de la Jurisprudencia aplicable al análisis del nivel inventivo de una solicitud de patente, se considera que una anterioridad o una combinación de dos anterioridades puede afectar dicho requerimiento de patentabilidad, si una persona medianamente versada en la materia encuentra enseñanzas o sugerencias claras que, a partir de un conocimiento medio en dicho campo, le permitan llegar de manera obvia a la

invención reivindicada. Adicionalmente se considera que una mejora sustancial, un efecto inesperado o una ventaja con respecto al arte previo es un indicio de nivel inventivo, por cuanto aporta un salto tecnológico en la regla técnica.

Respecto a lo anterior, el Tribunal de Justicia de la Comunidad Andina ha expuesto en la Interpretación Prejudicial del proceso 13-IP-2010:

> *"En este orden de ideas, se puede concluir que una invención goza de nivel inventivo cuando a los ojos de un experto medio en el asunto de que se trate, se necesita algo más que la simple aplicación de los conocimientos técnicos en la materia para llegar a ella, es decir, que de conformidad con el estado de la técnica el invento no es consecuencia clara y directa de dicho estado".*
> [señalado fuera del texto]

Por lo anterior, se entiende que para que una persona medianamente versada en la materia pudiera derivar de manera obvia una invención, requeriría simplemente aplicar sus conocimientos técnicos. En contraste, todo aquello que va más allá de la simple aplicación de los conocimientos técnicos y que no ha sido revelado en el estado de la técnica, debe ser considerado inventivo.

A este respecto, respetuosamente manifestamos nuestro desacuerdo con el análisis llevado a cabo por el Examinador y con las conclusiones del mismo y solicitamos atentamente la reconsideración de las razones por las cuales se objeta el nivel inventivo, con base en la siguiente información:

La presente invención difiere de D1porque incorpora un material granular que comprende un mineral de calcio dentro de un rango de concentración de PTH determinado dentro de una matriz de fibrina. Como se describe en las páginas 29 a la 31 y, en las Figuras 3 y 4 de la presente solicitud, esta composición mejora la reparación de defectos óseos, en particular las fracturas de huesos que se encuentran en nivel local. Con el fin de resaltar el campo técnico de la presente

invención, se han modificado las reivindicaciones 1 y 13 con el fin de indicar que las mismas están dirigidas "a la reparación de fracturas óseas".

Se presenta una copia de un artículo de revisión de Dempster *et al.*, Endocrine Reviews, 14(6): 690-709 (1993).

Este artículo explica en detalle el estado de la técnica de la investigación de PTH. En la página 69, columna izquierda, tercer párrafo, se describe un doble mecanismo regulador sobre el hueso con PTH ejerce un efecto inhibidor directo sobre los osteoclastos por una parte, y un efecto estimulante indirecto mediado por los osteoblastos en la otra parte.

La administración de PTH podría jugar un papel importante dando lugar a esos efectos, por ejemplo, cuando el PTH es aplicado intermitentemente en inyecciones diarias, se observó un incremento en hueso esponjoso, mientras que en la administración continua de PTH la absorción de hueso esponjoso parece ocurrir (página 696, párrafo D).

Debido al complejo modo de acción del PTH, los estudios conducidos por Dempster *et al.* no han llevado a respuestas directas. Además, debe señalarse que todos los resultados de Dempster fueron obtenidos por la administración sistémica de PTH, por ejemplo, por infusión o inyección dentro del cuerpo y pareció ser muy dependiente de la manera en la que fue administrado, el tejido óseo, la dosis y la co-administración de otros factores.

D1 es la primera publicación, en la que la administración local de PTH en el sitio de un defecto óseo se reporta para mostrar la formación de hueso en hueso esponjoso. D1 muestra esos efectos usando un modelo de detección descrito en Nuss *et al.*, BMC Muscoskeletal Disorders , 7:67-80 (2006).

En el modelo, los hoyos perforados de 8 mm de diámetro y 13 mm de profundidad son hechos en la región proximal y distal, tanto del húmero como del fémur. En este modelo, los defectos están rodeados por hueso esponjoso, por

ejemplo, los defectos son creados en huesos esponjosos con una capa delgada de hueso cortical en el borde de los defectos.

Este ambiente representa un lugar con alta actividad anabólica que no se expuso a ningún estrés mecánico. El modelo se usó como una herramienta de detección para evaluar la biocompatibilidad y la estimulación biológica de las sustancias analizadas. En la "Sección de Conclusión" los autores afirman que se trata de un modelo seguro para el cirujano experimentado, pues reduce el sufrimiento y el número de animales de experimentación, y sirve como una base excelente para probar los mejores materiales en un modelo animal más difícil, donde las propiedades mecánicas juegan un papel importante como en defectos óseos de tamaño crítico de largo, como el hueso maxilofacial o craneal.

En contraste, las "fracturas óseas" se definen en la presente solicitud como "una discontinuidad o ruptura a través de toda la estructura del hueso creando dos o más segmentos distintos del hueso" (página 7, tercer párrafo). Al contrario del modelo de ovejas perforadas, el modelo de defecto segmentado de 1 cm descrito en la presente solicitud representa un modelo animal mucho más difícil aprobado por cirujanos y autoridades regulatorias y necesario para obtener aprobación regulatoria para realizar ensayos clínicos en seres humanos.

A diferencia del modelo de la oveja perforada, el modelo de defecto segmentado de 1 cm (i) representa un modelo en el cual el sitio de efecto es inestable (el hueso se corta en dos piezas simulando la fractura) y por lo tanto, el hueso tratado se somete a un alto estrés mecánico durante la fase de reparación observada, (ii) representa un defecto mucho más grande que el del modelo de defecto perforado; (iii) representa un defecto que requiere una formación sustancial de hueso cortical y (iv) representa un modelo que requiere hueso de alta calidad que se forma de manera que el nuevo hueso es capaz de estabilizar el sitio de la fractura.

Dempster afirma que muchos agentes, tal como el fluoruro muestra el potencial para mejorar la masa ósea y aunque en general se acepta que el

tratamiento con fluoruro incrementa el volumen de hueso esponjoso en pollo, ratones, ratas, perros y cerdos, así como en humanos, que es difícil entre otros debido a la mala calidad del hueso en el que se forma (página 696, columna izquierda, segundo párrafo).

Teniendo en cuenta todos los resultados de PTH divergentes y poco claras reportado en el estado de la técnica (todos derivados de la aplicación sistémica), no era evidente en lo absoluto que una aplicación local de una formulación de PTH en un modelo de fractura real, aún si la formulación PTH/ fibrina/ gránulo de Ca mostrara actividad biológica en el modelo de detección del defecto de perforación en la oveja como indicativo de la efectividad en reparación de fracturas óseas sin presentar datos del modelo de fractura específica como el modelo de defecto segmentado de la oveja.

Además, se presenta una copia de un artículo científico de S. Boden ("Overview of the Biology of Lumbar Spine Fusion and Principles for Selecting a Bone Graft Substitute", SPINE, Volumen 27, No 16S, páginas S26-S31) para apoyar la opinión de que la capacidad de formación de hueso de una formulación, varía dependiendo del tipo de defecto y del mismo hueso, y que no se puede extrapolar los resultados de una localización anatómica a otra (página 29, columna izquierda, décima línea desde abajo): "Así, la validación de cualquier sustituto de injerto óseo en un sitio anatómico clínico no puede ser predictivo de su rendimiento en otra en otra localización."

A diferencia de la presente solicitud, el documento D1 no indica que la formulación descrita en el mismo podría ser para el tratamiento de fracturas óseas. En particular, no apunta hacia un rango de gran concentración de PTH o péptido de fusión de PTH.

El documento D2 no describe el uso de PTH o péptido de fusión de PTH y en particular no tiene una concentración adecuada para el tratamiento de fracturas óseas. De hecho, mientras que D2 menciona "defectos óseos" no se refiere a

fracturas óseas, como tal, o diferentes requisitos para diferentes tipos de tratamientos de defectos óseos como las fracturas óseas.

Considerando lo anterior, es claro que la revisión sistemática de las enseñanzas de D1 y D2 no permitirían a una persona con conocimientos medios en el estado del arte anticipar o obtener la presente invención sin haber desarrollado investigación científica adicional.

Lo anterior es importante, toda vez que el análisis problema-solución que, en opinión de la Guía del Despacho resulta obligatorio aplicar en estos caso, obliga a tener en cuenta una visión de conjunto de las anterioridades más que una simple enumeración de diferencias sin contexto técnico y que expresamente señala lo siguiente, con respecto al examen de nivel inventivo:

> "El examinador debe presentar los documentos relevantes que anulan el nivel inventivo de la invención en estudio y el examen de cómo las divulgaciones previas inducen a la persona del oficio normalmente versada en la materia a realizar la invención **sin que se hubiese requerido de su parte investigación alguna**".(página 71)

Por lo tanto, en el presente caso, con independencia de las enseñanzas puntuales que contengan los documentos D1 y D2, es claro que el análisis de conjunto de estos documentos no permitiría obtener de manera obvia la presente invención, en el sentido de que para obtener su resultado era obligatorio efectuar investigación adicional.

4.3 <u>Conclusión</u>

El documento D1, el documento D2 o su combinación, no resultan anterioridades válidas capaces de afectar el nivel inventivo del proceso de la presente solicitud.

Por lo tanto, consideramos que el Examinador está pasando por alto el hecho de que el inventor en el momento de solucionar el problema técnico de la solicitud tuvo que emplear habilidad inventiva, pues la presente invención no resulta de la mera extrapolación de las enseñanzas del arte previo, como se afirma. Se está desconociendo el mérito investigativo empleado y que está aportando una ventaja al arte previo.

Dicho mérito investigativo ha sido reconocido por otra oficina de patentes del mundo, la cual ha otorgado patente para una invención equivalente. Tal es el caso de Australia, patente No. AU2006204461 (B2).

5. Puesto que se ha dado respuesta a todas las objeciones formuladas por el Examinador, se solicita atentamente proceder al otorgamiento de la solicitud de patente. En este sentido, respetuosamente recordamos al Examinador que la patente no podrá ser negada con base en objeciones diferentes a las expuestas en la acción oficial No. 08752, toda vez que al no haber sido trasladadas a mi representada, dicha decisión violaría tanto el tenor literal del artículo 45 de la Decisión 486 como el derecho constitucional al debido proceso. En este evento, y con base en el inciso 2 del artículo 45, respetuosamente solicitamos al Examinador expedir una nueva acción oficial que permita a mi representada contra argumentar las mismas o modificar la solicitud con el fin de cumplir los requisitos de patentabilidad.

6. <u>Anexos</u>

- Nuevo pliego reivindicatorio conformado por 20 reivindicaciones
- Formulario de modificaciones en solicitud pendiente
- Recibo por concepto de diez (10) reivindicaciones adicionales a la décima por valor de $300.000
- Artículo: S. Boden. "Overview of the Biology of Lumbar Spine Fusion and Principles for Selecting a Bone Graft Substitute", SPINE, Volumen 27, No 16S, páginas S26-S31
- Artículo: Dempster *et al.*, Endocrine Reviews, 14(6): 690-709 (1993).

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- Artículo: Nuss *et al.*, BMC Muscoskeletal Disorders , 7:67-80 (2006).
- Artículo: Shen *et al.* Fibrin Gel Structure: Influence of Calcium. Biochemical and Biophysical Research Communications. Vol. 56(3): 793-798 (1974)

Señor Director,

LUZ CLEMENCIA DE PÁEZ
C.C. 35.456.344 de Usaquén
T.P.A. No. 23.555

COLO 16023 841 002 3
COLO-13936-843-001-9
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1. **Identificación del Tramite**

☐ **Error material** ☒ **Modificación a una Solicitud en trámite**

☒ Patente de Invención

☐ Patente de Modelo de Utilidad

☐ Registro de Diseño Industrial

☐ Esquema de Trazado de Circuitos Integrados

2. **Datos del solicitante**

Persona Natural ☐ Persona Jurídica ☒

BAXTER INTERNATIONAL INC. y BAXTER HEALTHCARE S.A.

Nombre o denominación / Nombre o razón social completa

Nombre representante legal

Tipo de empresa Micro ☐ Pequeña ☐ Mediana ☐ Otra ☐

Documento de identificación: ☐ C.C. ☐ C.E. ☐ NIT ☐ Otro Número_____

Nacionalidad/País de constitución	Dirección y domicilio del titular	Ciudad
Estados Unidos de América Y Suiza Respectivamente	One Baxter Parkway, Deerfield, Illinois 60015 Y Thurgauerstrasse 130 8152, Glattpark (Opfikon) Respectivamente	Deerfield Y Glattpark (Opfikon) Respectivamente

Dirección electrónica	No. Fax	Número telefónico
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☐ **Autorizo expresamente a la Superintendencia de Industria y Comercio para que todas las comunicaciones sean enviadas a través de la dirección de correo electrónico**

3. Datos del apoderado:

Apellidos y Nombre	Documento de identificación	Tarjeta profesional
De Páez Luz Clemencia	C.C. 35.456.344	T.P.A. 23.555

Dirección y domicilio		Ciudad
Carrera 4ª No. 72 –35		Bogotá
Dirección electrónica	**No. Fax**	**Número telefónico**
cavelier@cavelier.com	2 11 86 50	3 47 36 11
En caso de haber presentado poder general para asuntos que se adelanten ante la Delegatura de Propiedad Industrial, sírvase indicar el número de su radicación o protocolo de poder general		

4. Datos del trámite

Corrección:

Expediente No **07.080.236**

Aparece

Debe ser

Modificación:

☒ **Modificación al capítulo reivindicatorio.**
Presento nuevo capítulo reivindicatorio compuesto por 20 reivindicaciones y pido respetuosamente que el examen de patentabilidad sea realizado sobre estas nuevas reivindicaciones presentadas. Respetuosamente manifiesto que a pesar de eliminar materia del pliego reivindicatorio originalmente presentado, mi representada no renuncia a la protección que pueda obtener sobre dicha materia y se reserva el derecho de presentar una solicitud divisional que la contenga durante el trámite de la presente solicitud, beneficiándose de la fecha de prioridad inicialmente reivindicada.

☐ **Modificación al capítulo descriptivo.**

☐ **Modificación al resumen.**

☐ **Modificación a los dibujos.**

☐ **Modificación al solicitante.**

5. Anexos

- ☐ Poder, si fuere el caso.
- ☒ Documento que contiene las modificaciones o correcciones.
- ☐ Copia de la solicitud y sus anexos en formato magnético.
- ☒ Comprobante de pago por 10 reivindicaciones adicionales después de la décima reivindicación por $300.000

6. Firma	
Nombre y apellidos **LUZ CLEMENCIA DE PAEZ**	Firma Luz Clemencia de Paez
C.C. 35.456.344 DE Usaquén	Tarjeta Profesional 23.555 TP #23555 CSJ

SCT\SDC\WPCL3003\ID-217703

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REIVINDICACIONES:

1. Una formulación adecuada para la reparación de fracturas óseas que comprende

(i) un péptido seleccionado del grupo que consiste de PTH y un péptido de fusión PTH;

(ii) un material granular que comprende un mineral de calcio y

(iii) una composición capaz de formar una matriz de fibrina bajo condiciones fisiológicas que comprenden un componente precursor de firbinógeno y un componente precursor de trombina,

en donde el PTH o el péptido de fusión de PTH está presente en un rango de concentración de entre 0.01 a 2 mg/mL de matriz de fibrina o los componentes precursores que forman la matriz, con la condición de que una concentración de 0.4 mg/mL de matriz de fibrina o de los componentes precursores que forman la matriz no está incluida.

2. La formulación de acuerdo a la reivindicación 1, en donde el componente precursor de fibrinógeno o el componente precursor de trombina comprende además una fuente de calcio.

3. La formulación de acuerdo a la reivindicación 1 o 2, en donde el péptido de fusión de PTH comprende al menos dos dominios en donde el primer dominio comprende PTH y el segundo dominio comprende un dominio de sustrato reticulable.

4. La formulación de acuerdo a cualquiera de las reivindicaciones 1 a 3, en donde el PTH se selecciona del grupo que consiste de PTH_{1-84}, PTH_{1-38}, PTH_{1-34}, PTH_{1-31}, y PTH_{1-25}.

5. La formulación de acuerdo a la reivindicación 4, en donde el PTH es PTH_{1-34}.

6. La formulación de acuerdo a la reivindicación 3, en donde el segundo dominio comprende un dominio de sustrato de transglutaminasa.

7. La formulación de acuerdo a la reivindicación 6, en donde el dominio de transglutaminasa comprende un dominio de sustrato de Factor XIIIa.

8. La formulación de acuerdo a la reivindicación 3, en donde el péptido de fusión PTH comprende además un sitio de degradación entre el primero y segundo dominio.

9. La formulación de acuerdo a cualquiera de las reivindicaciones 1 a 8, en donde el material granular es una mezcla de fosfato de tricalcio e hidroxipatita.

10. Un kit que comprende la formulación de acuerdo a cualquiera de las reivindicaciones 1 a 9.

11. Un kit de acuerdo a la reivindicación 10, en donde el componente precursor de fibrinógeno y el componente precursor de trombina se separan uno del otro.

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12. El kit de acuerdo a la reivindicación 10 u 11, en donde el péptido de fusión PTH comprende además un sitio de degradación entre el primer y segundo dominios, dicho sitio de degradación es un sitio de degradación enzimático o hidrolítico.

13. Una matriz complementada adecuada para la reparación de fracturas óseas que comprende

(i) PTH o un péptido de fusión PTH;

(ii) un material granular que comprende un mineral de calcio y

(iii) fibrina;

En donde dicho PTH o péptido de fusión PTH está presente en un rango de concentración de entre 0.01 a 2 mg/mL de matriz de fibrina, con la condición de que la concentración de 0.4 mg/mL de matriz de fibrina no está incluido.

14. La matriz complementada de acuerdo a la reivindicación 13, en donde el PTH se selecciona del grupo que consiste de PTH_{1-84}, PTH_{1-38}, PTH_{1-34}, PTH_{1-31}, y PTH_{1-25}.

15. La matriz complementada de acuerdo a la reivindicación 14, en donde el PTH es PTH_{1-34}.

16. La matriz complementada de acuerdo a cualquiera de las reivindicaciones 13 a 15, en donde la matriz complementada se forma de una composición capaz de formar una matriz de fibrina y un péptido

de fusión, que comprende el PTH en un primer dominio, y un dominio de sustrato covalentemente reticulable en un segundo dominio.

17. La matriz complementada de acuerdo a la reivindicación 16, en donde el segundo dominio comprende un dominio de sustrato de Factor XIIIa.

18. La matriz complementada de acuerdo a cualquiera de las reivindicaciones 13 a 17, en donde el péptido de fusión PTH comprende además un sitio de degradación entre el primer y segundo dominios.

19. La matriz complementada de acuerdo a la reivindicación 18, en donde el sitio de degradación es un sitio de degradación enzimático.

20. La matriz complementada de acuerdo a cualquiera de las reivindicaciones 13 a 19, en donde el material granular es una mezcla de fosfato de tricalcio e hidroxiapatita.

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An animal model in sheep for biocompatibility testing of biomaterials in cancellous bones

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Abstract

Background: The past years have seen the development of many synthetic bone replacements. To test their biocompatibility and ability for osseointegration, osseoinduction and -conduction requires their placement within bone preferably in an animal experiment of a higher species.

Methods: A suitable experimental animal model in sheep with drill holes of 8 mm diameter and 13 mm depth within the proximal and distal humerus and femur for testing biocompatibility issues is introduced.

Results: This present sheep model allows the placing of up to 8 different test materials within one animal and because of the standardization of the bone defect, routine evaluation by means of histomorphometry is easily conducted. This method was used successfully in 66 White Alpine Sheep. When the drill holes were correctly placed no complications such as spontaneous fractures were encountered.

Conclusion: This experimental animal model serves an excellent basis for testing the biocompatibility of novel biomaterials to be used as bone replacement or new bone formation enhancing materials.

Background

The use of resorbable and non- resorbable biomaterials, such as hydroxyapatite or tricalcium phosphate, as synthetic bone replacements is well established in orthopaedic, maxillofacial and dental surgery [1,2]. Although autologous cancellous bone is the material of choice for bone replacement and induction there are limitations in relation to its use, such as limited amount of material, additional surgical procedure, prolonged surgery and complications of wound healing at the donor site. Because of this there is continuous interest in the development of new synthetic materials [3-5].

The most important aspect in the development of new (biodegradable) biomaterials is the experimental and clinical testing for biocompatibility [6-8]. This is closely followed by the bioactivity, which relates to the resorption or integration of the implanted material into the surrounding bone, also called "osseointegration" and the ability to initiate and support the apposition and integration of the new bone, in particular in comparison with previously established materials [4].

Various animal species are used for these biocompatibility tests, such as the mouse [9], rat [10-18], guinea pig [19],

Figure 1
The photograph shows the 8 mm special depth-regulating bit and corresponding drill guide (Ia) and the close-up (Ib) the flattened tip of the drill bit.

rabbit [20-23], dog [24-28] goat [29] and sheep [30-32]. Furthermore, different implantation sites and methods have been used to examine biocompatibility issues. Among them are intra-peritoneal [13], subcutaneous [12,14,17,33], intraosseous such as in the mandible [19,27], femur [11,15,24,25]), tibia [18,20,21,23,26,30], cranial bone [28,31] and intramuscular [9,16,29] applications.

The use of sheep for orthopaedic research continues to increase. This is due to the similarities with humans in weight, bone and joint structure and bone regeneration [34-36]. Although rodents may be less expensive, they have different bone morphology. Another practical reason is that rodents often are too small in size to test degradable materials in bone especially in combination with internal fixation and fracture repair [37,38]. Furthermore, the influence of the different mechanical properties of bone from different species awaits clarification in relation to the outcome of these studies [39,40].

To contribute to the standardisation of testing new and biodegradable materials for use in orthopaedics, maxillofacial and dental surgery our research group has developed an animal model with sheep that allows the intraosseous implantation of 8 different samples per sheep in long bones [41,42]. This animal model facilitates testing inter- and intra-individual differences among different materials while at the same time reducing overall suffering of animals as well as necessary numbers to satisfy statistical requirements. It has been already successfully applied for several studies related to testing biodegradable materials [32] and to the knowledge of the authors has never been described before in the literature.

Methods

Instruments

In addition to routine surgical instruments, Weitlaner and Gelpi-retractors, a periosteal elevator, pneumatic drill (Synthes, Waldenburg, Switzerland) with an 8 mm, slightly modified drill bit (KaVo INTrASurg 500®, KaVo Dental AG Biberach, Germany, modified by Synthes, Waldenburg, Switzerland) and a corresponding drill guide were used (Fig. 1). The drill bit was modified with a special depth-regulating device and the tip was flattened while still having good cutting characteristics.

Biomaterials

Implanted were various cements (β-tricalciumphosphates, brushite, hydroxyapatite), hydrogels (fibrin-based, polyethylene glycol) and other resorbable or non-resorbable bone replacement materials as composites with or without growth factors such as parathyroid hormone (PTH_{1-34}), bone morphogenic protein (BMP-2), transforming growth factor (TGFβ) and insulin-like growth factor 1 (IGF-1).

Experimental animals

Used were 70 healthy ewes, White Swiss Alpine Sheep, with an age of 2 to 5 years and a bodyweight of 53 to 80 kg. The implantation sites were the proximal part of the diaphysis and distal epiphysis of humerus and femur (Fig. 2). This provided a total of 8 implant sites per animal. All animal experiments were carried out according to the Swiss Laws of animal welfare and were approved by the Ethics Commission of the official veterinary authorities (Authorisation Numbers: 53/2000, 139/2000, 09/2002, 176/2003, 118/2004, 14/2005, 15/2005, 90/2005).

The experimental animals were kept in stalls prior to surgery. Food was withdrawn 36 hours, and water 6 hours prior to anaesthesia. A pre-anaesthetic examination was performed including haematology and a chemscreen consisting of liver enzymes, urea, creatinine, protein, albumin, sodium and potassium. After recovery of the animals from anaesthesia, they were initially kept in small groups

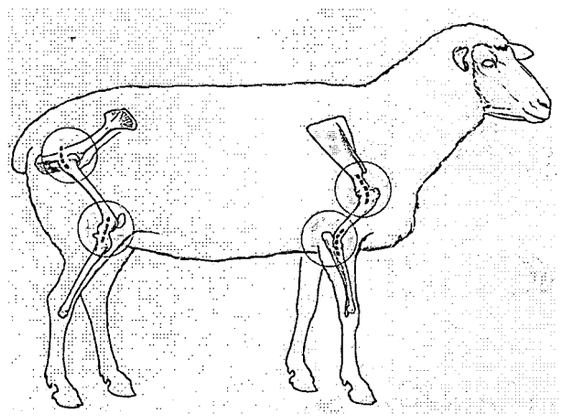

Figure 2
Layout of the approaches to the implantation sites: the dotted lines represent the sites of skin incisions.

in stables. After 10 days, the skin staples were removed and the animals were allowed to join the flock on the fields normally at 20 days after surgery. They were checked daily for lameness and additional clinical problems. Food and water were given ad libitum.

Surgery

Animals were sedated with medetomidine (5 μg/kg BW, Domitor™, Orion Pharma Animal Health, Finland) and anaesthesia was induced with ketamine (2 mg/kg BW, Narketan® 10, Chassot GmbH, Germany) in combination with diazepam (0.01 mg/kg BW, Valium®, Roche, Switzerland) and maintained with 0.8 Vol% isoflurane (Forene®, Abbot AG, Switzerland) in O_2 and an infusion of Ringer's solution with 60 mg ketamine (Narketan™ 10, Chassot GmbH, Germany)/litre at a rate of 10 ml/kg BW/hour. The animals received as pre-and post-operative prophylaxis 30,000 IU penicillin/kg BW (Hoechst AG, Germany) and 6 mg gentamicin/kg BW (Streuli & Co AG, Switzerland) intravenously twice a day. In addition, they received subcutaneously 500 Units of equine tetanus serum as a single application (Tetanus Serum Veterinaria AG, Zurich, Switzerland).

Analgesia was maintained through injection of 0.01 mg buprenorphine/kg BW i.v. perioperatively and additionally 4 mg carprofen/kg BW i.v. (Rimadyl®, Pfizer Inc., NY, USA) postoperatively for 3 days. The area in the region of the incisions was clipped and disinfected in the standard manner.

The animals were placed in left or right lateral recumbency on the operating table with the limbs placed in a horizontal position. The approach to the bone was always from the lateral side (Fig. 2). It is important for the forelimb that the humerus is positioned parallel with the humeral condyles being at a 90° angle to the surgery table. Furthermore the limb should be slightly flexed to the position that the lateral epicondyle of the humeral condyle can be palpated easily directly under the skin. The hind limb should be also slightly flexed such that the lateral collateral ligament can be palpated through the skin, although a slight downward inclination of the hind limb may facilitate access to the proximal femur. Both, the fore- and hind limbs should be additionally supported from underneath so that they cannot be pushed down when drilling the hole but remain stable in the prepared plane.

With all approaches an attempt was made to keep the skin incisions and preparation of soft tissue down to the bone as small as possible (ca. 6–8 cm in length). To facilitate the further surgical approaches to all locations, the wounds were kept open by means of either a Weitlaner retractor alone or in combination with a Gelpi retractor that was usually placed perpendicular to the already placed Weitlaner retractor (proximal and distal femur). The periosteal elevator was used to prepare the location of the drill hole such that all overlying soft tissues were removed from the bone. This facilitated the placement of the drill guide and avoided the slippage of the drill bit initially before the drill had started to penetrate the cortical bone. The description of the four different surgical sites is given below. A total number of 560 drill holes were placed (n = 560 with 70 drill holes in each location). Immediately after drilling, the holes were filled with the various test materials. After filling the drill holes, the different wound layers as described in the approaches were closed separately with non-resorbable suture material (Vicryl 2/0, Johnson & Johnson, Brussels, Belgium) and the skin was stapled (Davis and Geck Appose ULCr, B. Braun Aesculap AG, Tuttlingen, Germany)

Approaches

Proximal part of humeral diaphysis (Fig. 3)

The skin incision began at the level of the acromion and followed a slightly curved line over the shoulder joint to the middle of the proximal third of the humerus (Fig. 3a). After dissecting the subcutaneous fascia the acromial part of the deltoid muscle was visible and situated between the trapezius and the omotransversarius muscles. These muscles were then retracted caudally (Fig. 3b). Beneath appeared the tendon of insertion of the infraspinatus muscle. A hole was then drilled into the humerus caudally to this tendon and in the medial direction. A small vessel without a given name of less than 1 mm in diameter and

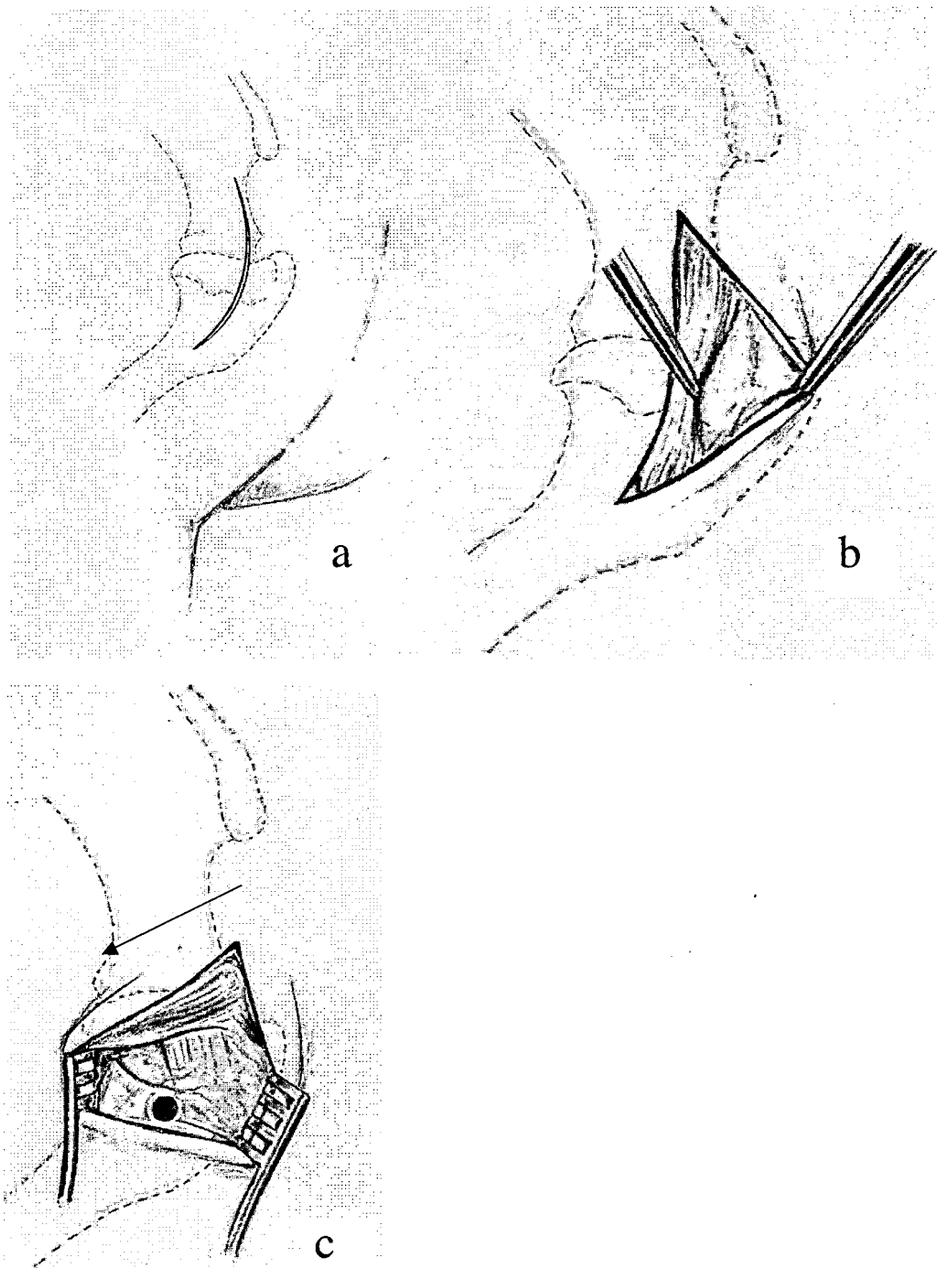

Figure 3
Surgical procedure at the proximal diaphysis of the humerus: The skin incision extends from the acromion to the distal end of the tuberculum majus (Fig. 3a). The acromial part of the deltoid muscle is dislocated caudally to expose the tendon of insertion of the infraspinatus muscle (Fig. 3b), where the hole is drilled caudally to the insertion point and distally to the unnamed vessel (arrow) (Fig. 3c).

crossing the bone directly above the insertion of the tendon served as a consistent landmark (Fig. 3c).

Distal epiphysis of the humerus (Fig. 4)

A slightly curved skin incision was made laterally, directly over the lateral epicondyle of the humerus (Fig. 4a). After the dissecting of the subcutaneous and deep fasciae the lateral epicondyle giving origin to the lateral collateral ligament could be palpated. An incision was made in the ligament paralleling the direction of its fibres (Fig. 4b). The drill guide was passed through the opening and the drill hole situated exactly in the centre of the groove and the lateral humeral condyle (Fig. 4c).

Proximal part of the femoral diaphysis (Fig. 5)

The skin incision was made directly above the easily palpable greater trochanter of the femur (Fig. 5a). The medial gluteal muscle appeared immediately after the incision of the subcutaneous fascia. The muscle was penetrated in a longitudinal direction using a blunt incision (Fig. 5b). The underlying fascia lata was opened with a scalpel blade exposing the lateral vastus of the quadriceps muscle (Fig. 5c). The access to the bone was then made directly beneath the greater trochanter through a longitudinal incision of the lateral vastus of the quadriceps muscle (Fig. 5d). The drill guide was placed perpendicular to the bone axis and firmly pressed to the bone to avoid slippage during drilling. It was located 1.5 cm below the tip at the base and in the centre of the greater trochanter (Fig. 5e).

Distal epiphysis of the femur (Fig. 6)

Palpable bone landmarks are the patella, the tibial tuberosity and the lateral condyles of the femur. The skin incision was performed in the triangle between the patella and the tibial tuberosity on the one and the lateral condyles of femur and tibia on the other side (Fig. 6a). With the splitting of the subcutaneous fascia the aponeurosis of the biceps muscle was visible. The muscle fascia was opened with a small (ca. 2 cm) incision perpendicular to the muscle fibres (Fig. 6b) and the underlying muscle tissue was bluntly dissected down to the lateral condyle of the femur. The hole was drilled closely to the proximal origin of the lateral collateral ligament of the stifle joint (Fig 6c).

After surgery the exact locations of the material-filled holes could be determined using radiographs (Figs. 7a and 7b). Due to the different densities of the overlying soft tissues (pelvis and proximal humerus versus elbow and stifle joint), each radiograph had to be taken separately amounting to 8 radiographs per animal. If radiographic follow-ups had to be obtained at several time intervals, each time the sheep had to be subjected to a short anaesthesia (Sedation with medetomidine (5 μg/kg BW, Domitor®, Orion Pharma Animal Health, Finland) and induction with ketamine (2 mg/kg BW, Narketan® 10, Chassot GmbH, Germany) in combination with diazepam (0.01 mg/kg BW, Valium®, Roche, Switzerland) and maintained with propofol (2–4 mg/kg BW, Disoprivan® Astra Zeneca, Switzerland) to assure appropriate quality of the radiographs.

Evaluation of samples

After the sheep were sacrificed both humeral and femoral bones were harvested immediately, freed from all overlying soft tissues and, if possible, the drill hole in the lateral cortex was identified. Time points of sacrifice varied from 2, 4, 8, 16 to 24 weeks depending on the original study question. The long bones were radiographed again using a faxitron machine (Cabinet x-ray-faxitron series, model 43855A, Hewlett Packard®, USA) that allows visualizing the detailed bone structure of the trabecular bone. Thereafter, bone blocks were cut using a bone saw (K 410, Kolbe GmbH, Elchingen, Germany) assuring a rim of at least 3–4 mm of adjacent bone at the side and at the bottom of the original bone defect. Care was taken that the blocks were sectioned parallel to the original bone cylinder (Fig. 8). If important in relation to the original study question (e.g. fine trabecular structure within bone defect or adjacent bone) the blocks were radiographed again with the faxitron machine (Fig. 9) or they were subjected to micro-computed tomography. All samples were fixed in either 4% paraformaldehyde or 10% fresh, buffered formaldehyde, before they proceeded for histology of non-decalcified bone section as described elsewhere [42,43]. Briefly, after fixation, samples were washed in buffered saline, dehydrated in a series of alcohol, defatted in xylene under vacuum, infiltrated in methylmethacrylate (methacrylacid-methylester; dibuthylphtalate and perkadox in a proportion 89,5: 10: 0,5) and then finally embedded in the same solution using special Teflon molds placed in a standard water bath at 30°C. Ground sections (30–40 μm) and thin sections (5 μm) were prepared using special equipment (Leica® SP 1600 and Leica® RM 2155; Leica Instruments GmbH, Nussloch, Germany). Sections were either surface stained with toluidine blue in case of ground sections, or deplastified with Methoxyethyl-acetate (Merck AG, Switzerland) and stained with either toluidine blue or von Kossa/McNeil in case of thin sections. Before mounting the ground sections to the opal, acrylic Plexiglas slides (Wachendorf, Perspex GS, Acrylicglas Opal 1013) microradiographs were taken to visualize the stage of calcification of the bone samples within and adjacent to the biomaterial (Fig. 10). Sections were cut in the middle of the bone defect and perpendicular to the original drill hole revealing the round original bone defect for further histological assessment with light microscopy. Ground sections were used to determine resorption of biomaterials, new bone formation and bone remodelling of the adjacent bone, but also to measure the area and

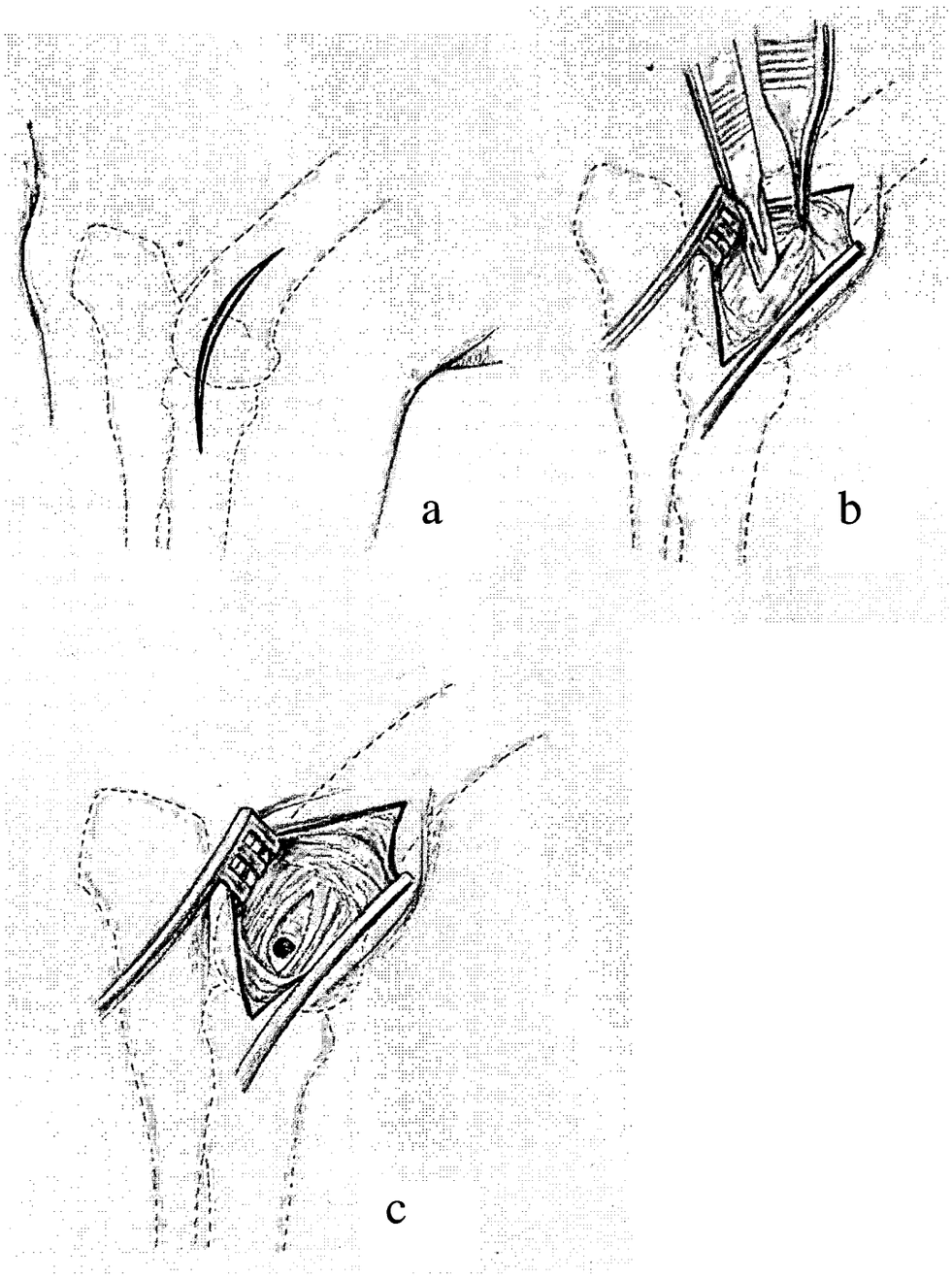

Figure 4
Surgical procedure at the distal epiphysis of the humerus: The skin incision is performed directly above the lateral epicondyle of the humerus (Fig. 4a). Then the proximal part of the Lig. collaterale laterale is incised with a scalpel following the direction of the fibres. The forceps tip is on the dorsal and prominent rim of a groove that gives origin to this ligament (Fig. 4b). The drill hole is situated directly in the centre of the groove between the fibres of the lateral collateral ligament (Fig. 4c).

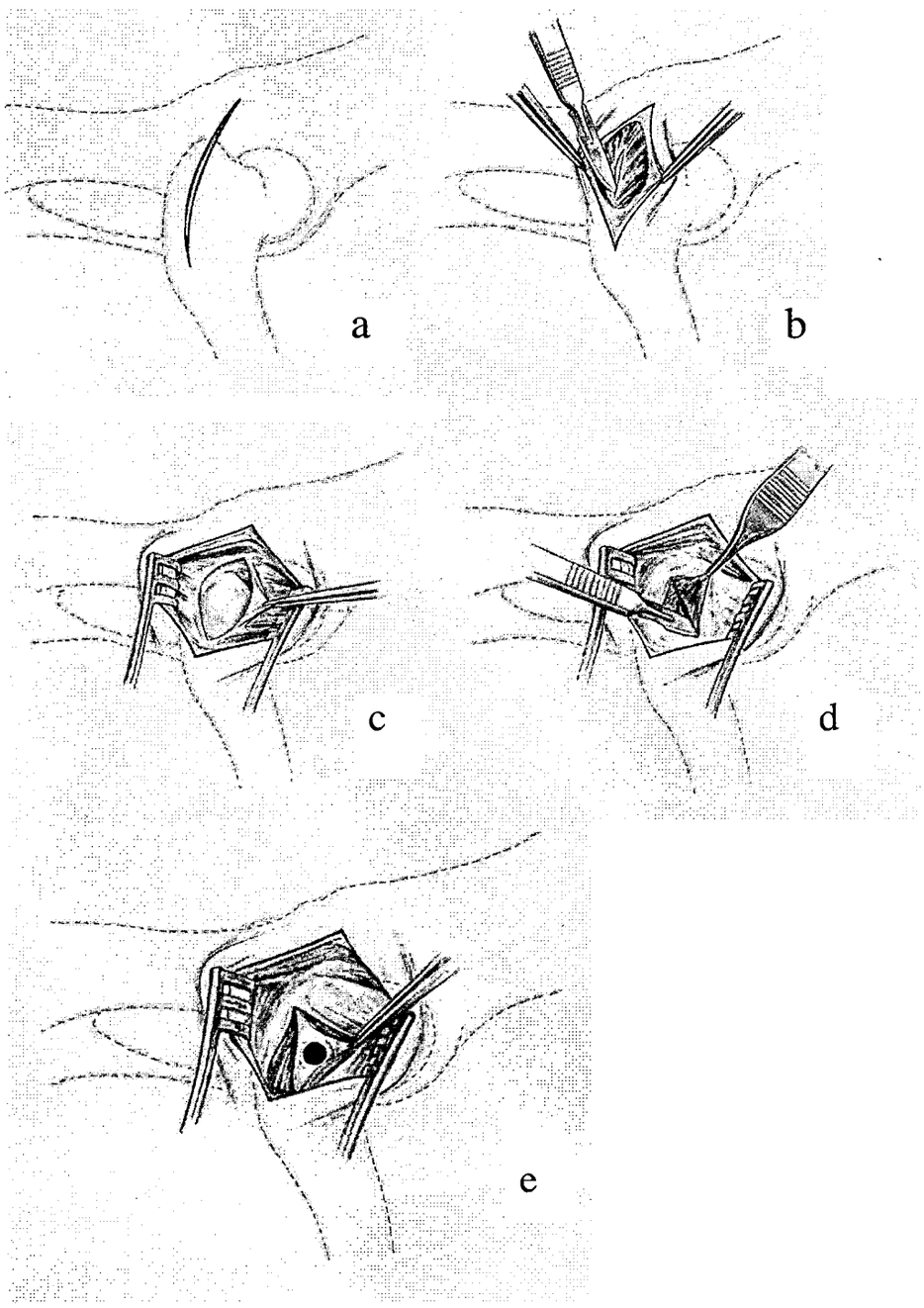

Figure 5
Surgical procedure at the proximal part of the femoral diaphysis: The skin incision is situated directly above the greater trochanter (Fig. 5a). The incision in the deep layer of the fascia lata reveales the medial gluteal muscle that is separated longitudinally (Fig. 5b) to expose the lateral of the quadriceps muscle (Fig. 5c). The lateral vastus is split longitudinally (Fig. 5d) and the final drill hole is inserted in the centre at the base and 1.5 cm distally to the tip of the greater trochanter (Fig. 5e).

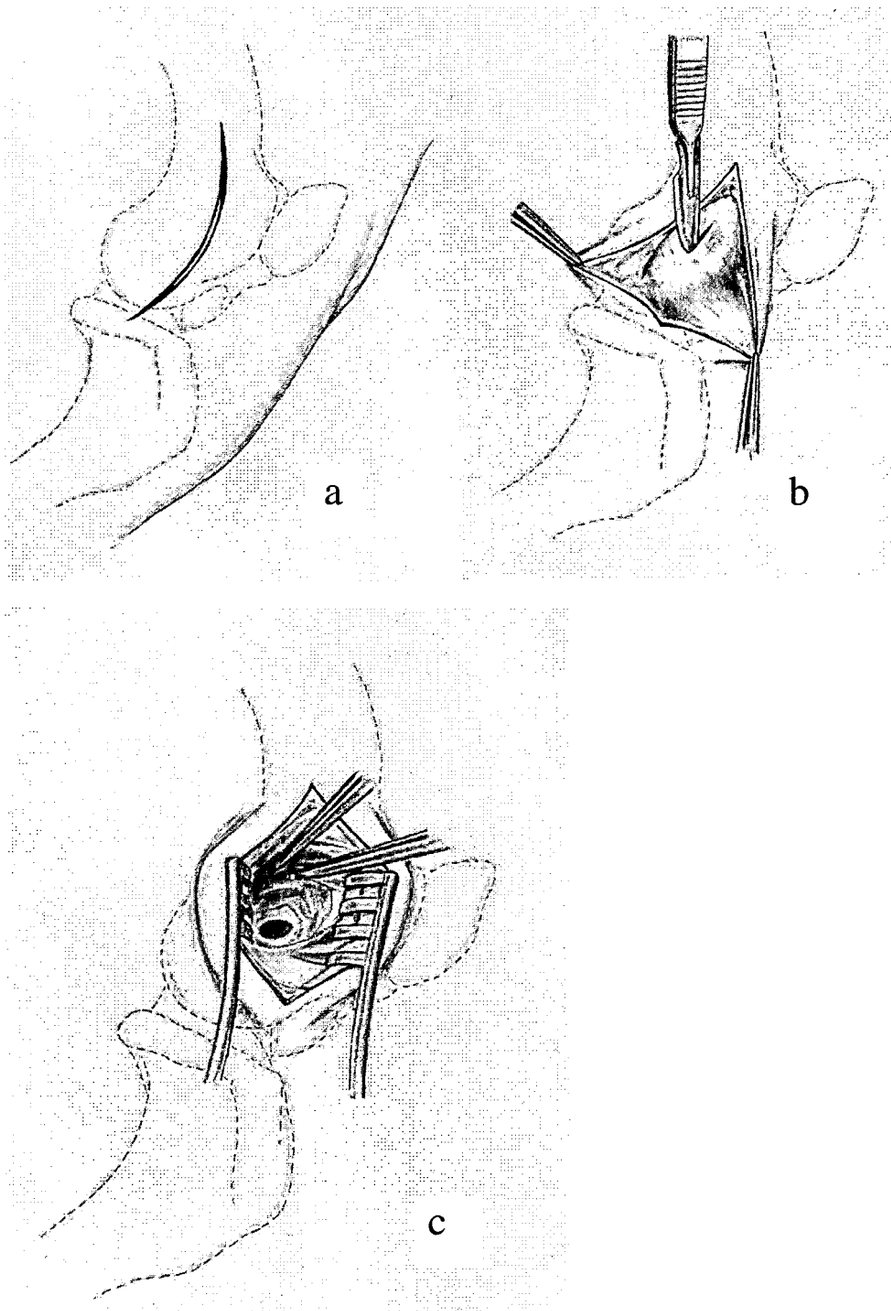

Figure 6
Surgical procedure at the distal epiphysis of the femur: The skin incision extends between the lateral femoral condyle and the tibial tuberosity of the tibia (Fig. 6a). The incision in the aponeurosis of the biceps femoris muscle (Fig. 6b) is made perpendicular to the muscle fibres of the biceps femoris muscle and the drill hole is inserted just proximal to the origin of the lateral collateral ligament of the stifle joint (Fig. 6c).

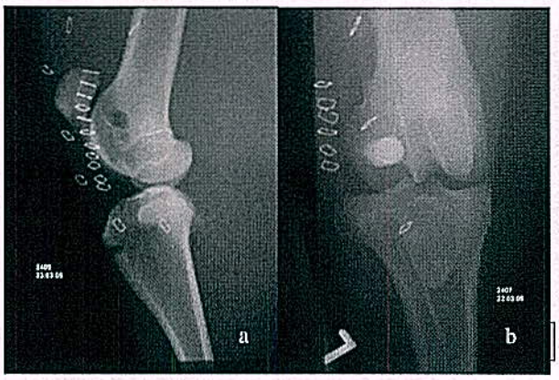

Figure 7
Postoperative lateromedial (Fig. 7a) and craniocaudal (Fig. 7b) radiograph of the distal part of the left femur after the injection of two specific, but different biomaterials. While bone cement (Fig. 7b) reveals immediate radiodensity, a biomaterial based on hydrogel and without radiopacifier cannot be visualized immediately after surgery. If bone formation takes place over time, radiodensity will increase gradually.

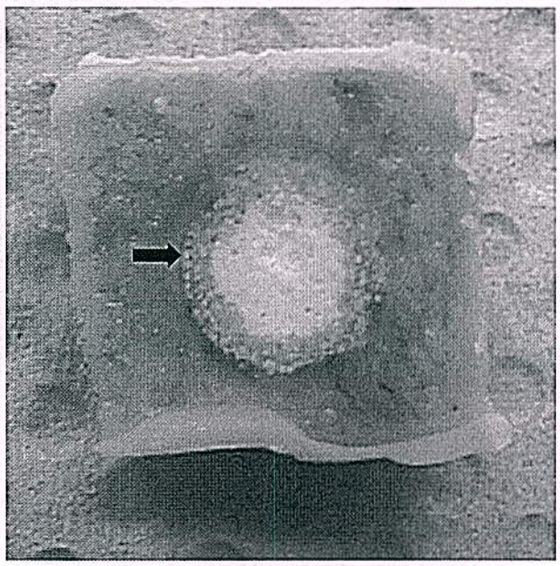

Figure 8
Preparation of the bone blocks after sacrifice: Note the rim of adjacent bone (arrow) around the original bone defect filled with biomaterial.

percentage of bone, fibrous tissue and remnants of biomaterials within the original defect by means of histomorphometry (Leica Qwin®, Leica Quips®, Leica, Glattbrugg, Switzerland). For this, pictures were captured with the macroscope as digital images in TIF-format (DC 200, Leica. Glattbrugg, Switzerland) and at a 5.8 times magnification. Thin sections were suitable for semi-quantitative evaluation of samples with the light microscope (Leica, DMR, Glattbrugg, Switzerland), where cellular reactions were assessed based on a semi-quantitative score that was specifically developed for each individual study [43].

Results

Anaesthesia and recovery phases were uneventful for 51 of the 55 animals. As soon as the sheep were awake they were placed in straw-lined small stalls. Ten days later, after removal of the skin staples, animals were released to larger stalls and finally were allowed to roam free on pasture after 20 days. At no time did they show signs of lameness or other discomfort.

For 4 sheep, the positioning of the drill holes was not exactly in the correct places described above, but slightly off towards the diaphysis of the femur or distal humerus respectively. These animals suffered either from a comminuted fractured humerus (n = 1) or femur (n = 3) within the first 3 postoperative days. All four animals were immediately euthanized after the complications occurred. All other drill holes (total of n = 528 holes) could be easily placed except in the distal humerus, where in 9 instances the drill hole was initiated too far cranially and distally resulting in slippage of the drill bit into the cranial pouch of the elbow joint including an incomplete drill hole within the cranial aspect of the lateral humeral condyle. In those 9 instances, where initial slippage of the drill bit occurred from the lateral humeral condylus, the surgeon gave up drilling the hole to avoid spontaneous fractures of the distal humerus. There, the biomaterials were never applied.

After the pre-planned time period the animals were slaughtered and the treated bones removed. Depending on the time point of sacrifice the original drill hole was easily detected or especially at later time points almost healed, at least at the cortical site. But even then, there was a small amount of connective tissue visible in the area indicating the original defect within the cortical bone. No macroscopic signs of inflammatory reactions were generally seen even at the early time points. However, this was dependent on the implanted material [44]. Normally, the radiographs taken with the faxitron machine revealed the location and direction of the drill hole, although at later time points (24 weeks) depending on the biomaterials bone healing could be so far advanced that detection proved to be difficult and only signs of intensive bone remodelling indicated the original bone defects.

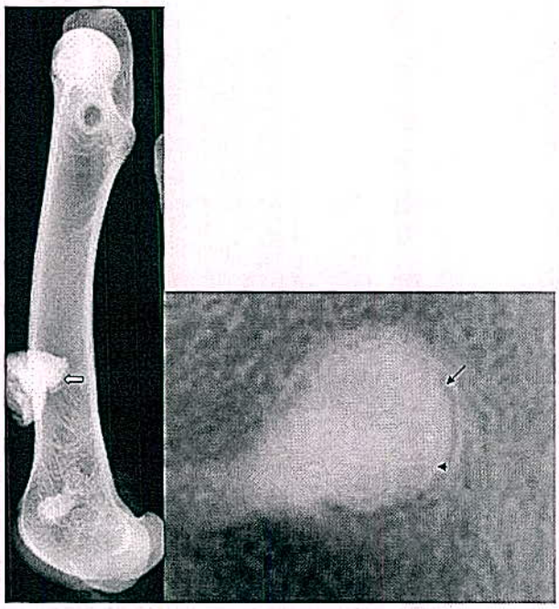

Figure 9
Radiograph taken with the faxitron machine: The technique, where the entire bone or bone samples already cut in blocks are radiographed with the faxitron machine, allows the assessment of bony changes, such as bone resorption (arrow head) or bone sclerosis (arrow) as a means for biocompatibility of the inserted biomaterials. In *Fig. 9a* an entire femur is shown (arrow: Bone-fixation device), whereas in *Fig.9b* only the original defect with a rim of adjacent bone is pictured.

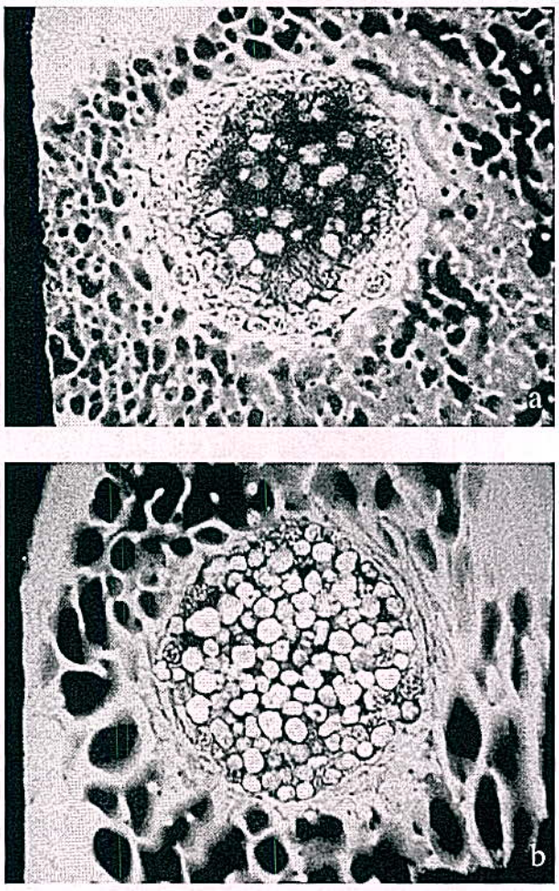

Figure 10
Microradiographs taken from two different biomaterial samples: Microradiographs show the stage of calcification of the bone after the application of bone enhancing materials (Fig. 10a) or both, remnants of bone cements based on calcium phosphate and replacement by new bone (Fig. 10b).

The bone samples could be easily embedded in acrylic resin and in all instances the size of samples allowed the preparation of non-decalcified histology sections for ground and thin sections. Depending on the biomaterials and stage of osseointegration or resorption the sectioning of thin sections required great skill from our histology technician. This was especially true for sections containing calcium phosphate bone cements where the biomaterial was brittle and if no bone replacement had taken place yet in the early sections (2, 4 and 8 weeks) the material simply disintegrated during sectioning. However, this hardly occurred in the ground sections (30 – 40 μm). There, the defect could be clearly distinguished from the surrounding bone. Thin sections allowed distinguishing cellular events in all instances even if the bulk of biomaterial was fallen off during sectioning since the immediately adjacent tissue could still always be evaluated (Fig. 11).

The time frame up to 6 months proved to be adequate for this size of defect, since most of the biomaterials tested were resorbed and replaced by new bone to at least two thirds of the original bone defect within this period. The same was true for the assessment of cellular events within this given time point. Degradation mechanisms could be easily followed at the interface between biomaterials and host tissue. The early time points (2, 4 weeks) were helpful to test biocompatibility issues since the appearance of foreign body or other mononuclear cells indicative of host reaction to the material occurred mainly within this early phase.

Discussion

The drill hole model in sheep as presented is well suited to test biocompatibility issues regarding biodegradable materials, but also to answer questions related to material resorption, substitution with new bone or less functional

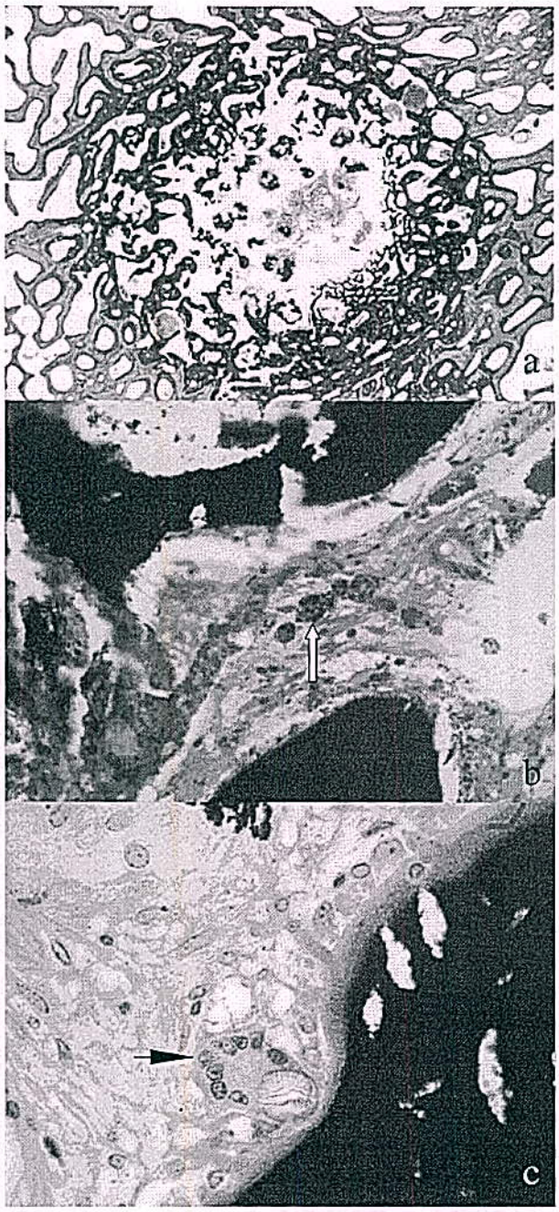

Figure 11
Histology samples: While ground sections (30–40 μm, surface stained with toluidine blue) are well suited for the assessment of osseointegration, new bone formation, materials resorption and histomorphometrical measurement (Fig. 11a), thin sections allow assessing cellular reactions such as degradation and elimination of biomaterials through macrophages (Fig. 11b, arrow) or the appearance of foreign body cells (Fig. 11c, arrow).

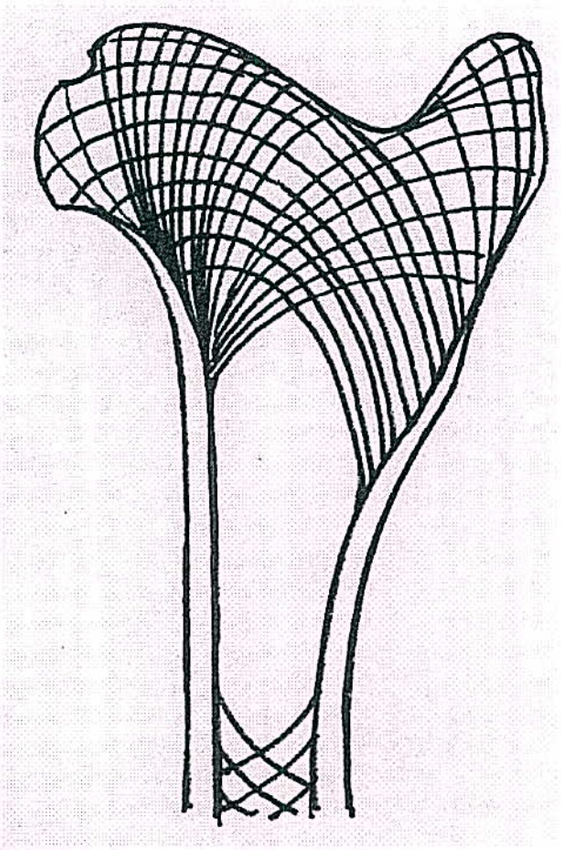

Figure 12
The graph represents a drawing of a longitudinal section of the proximal part of the femur. The lines picture the area where cancellous bone can be found, but also the trajectories indicating the direction of the mechanical tension lines in the bone.

tissue such as fibrous tissue. The experimental model was established in 70 sheep with a total of 560 drill holes for materials to be tested in bone. Several advantages are pertinent to this well standardized animal model with animal welfare being one of the most important aspects, closely followed by the possibility to translate results relatively easy to humans without the necessity of additional animal experiments related to biocompatibility questions.

The surgical technique requires a modified drill bit with a flattened tip, solid anatomical knowledge and experience of the surgeon. The small stab incisions and minimal preparation of soft tissues to access the bone cortex, where the drill hole will be placed, are part of the regimen of minimally invasive surgery and thus, atraumatic tech-

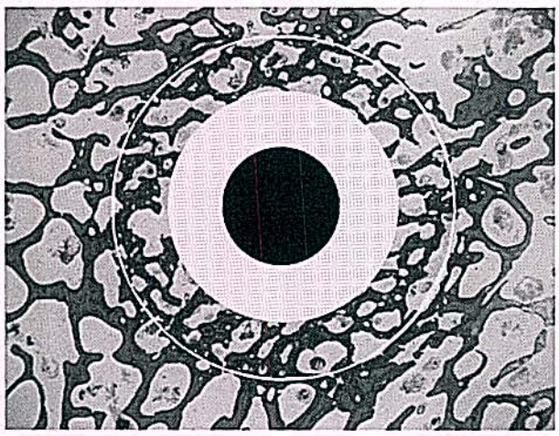

Figure 13
The picture shows the division in peripheral, middle and centre area of the bone defect as may be necessary to compare bone formation and biomaterials resorption over time or between different materials.

nique including prevention of unnecessary suffering of the animals.

The diligent and correct positioning of the limbs for surgery turned out to be a decisive factor for positioning the drill holes, especially for the distal humerus and femur. This allows optimal positioning of the drill hole that needs to be placed exactly as described in the surgical technique because of the quality of the brittle sheep bone. If the drill holes are slightly misplaced spontaneous fractures occur as described with the 4 animals with complications. The special structure and morphology of long bones in sheep corresponds to the function and mechanical load of the specific bone area [45]. This leads to a relatively large area of cancellous bone with a thin cortex in the metaphysis and epiphysis of the humerus and femur. Here, the mechanical pressure can be transferred to the cancellous network, while closer to the diaphysis this is not possible any longer since there is only cortical bone and fatty bone marrow to withstand the mechanical load (Fig. 12). The relative large drill hole of 8 mm in relation to the overall bone diameter obviously disturbs the tension lines of the bones significantly and gives rise to spiral fissures and cracks along these so called trajectories. This occurs even if only one cortex is drilled and is usually associated with severely comminuted fractures. If the drill holes are correctly placed within the metaphysis (proximal humerus and femur) or epiphysis (distal humerus and femur) this complication can be avoided. Especially in the distal humerus it may be better to stay away from drilling the hole if in doubt whether the position is correctly chosen. When the method was performed correctly, as described above, then there were no unforeseen fractures. In 9 cases the drill bit slipped into the cranial pouch of the elbow joint. To avoid spontaneous fractures, the drilling was not repeated and no biomaterial was applied. Since we only touched the joint but never penetrated any important structures, it had no clinical implication such as haematoma, pain or lameness.

Histology with non-decalcified bone sections proved to be suitable to assess bone healing as well as the behaviour of biodegradable materials. By sectioning the bone samples in midlength and perpendicular to the original drill hole the stage and ratio of new bone formation versus material resorption could be optimally visualized and calculated at its most remote location. Since any biomaterial used in bone finally will be replaced by creeping substitution [46] it may be safely assumed that if the centre and middle of the original biomaterial is replaced with new bone the rest of the original bone defect has already been replaced and is already undergoing bone remodelling. If deemed necessary serial sections can be prepared throughout the entire original defect, although the extensive workload and costs associated with preparing sections of non-decalcified bone may prove inhibitive. The standardized drill hole defect is well suited for histomorphometrical measurements where an automatic software program with the appropriately established "macro" allows the calculation of area percentages of bone, remnants of biomaterials as well as replacement with fibrous tissue. Apart from the overall percentage of each component the area may be divided in rings facilitating the comparison of the peripheral, middle and more central areas of the original bone defect [43]. This is interesting especially if the rate of resorption over time or between different biomaterials should be compared (Fig. 13).

It is common belief that for ethical reasons small experimental animals, such as mice, rats or rabbits, should be used wherever possible. Another reason to turn to small rodents is for economical aspects as rats and mice are much less expensive and easier to maintain as sheep. Biocompatibility of novel materials is often tested in less demanding animal models in the literature. Biological materials implanted into soft tissue, for example intramuscularly [9,16,29] or subcutaneously [12,14,17,33]), may answer questions pertaining to the local cellular and humoral reaction, but not to the degree of new bone formation, osteoinduction and -conduction. Even the local cellular degradation mechanisms may be different in soft and osseous tissue, since osteoclasts often involved in synthetic hydroxyapatite-based bone materials, are only present in bone but not subcutaneous or muscular tissues. In addition, any material placed in soft tissue may elicit the formation of a soft tissue capsule in an attempt to wall it off. This reaction most likely has nothing in common

with a response related to biocompatibility, but could also be a reaction to mechanical instability and different stiffness and rigidity of soft tissue and the implanted biomaterial. In the author's judgement the subcutaneous and intramuscular pouch model in rodents as introduced by Urist et al [47,48] is a valuable animal model for heterotopic osteoinduction triggered by biomimetic substances, but not for testing biocompatibility issues related to bone.

There are successful reports with drill holes used in rabbit tibias [23,26] and rat femurs [11]. Rodents may require less time and cost as well as less sophisticated surgery and anaesthesia facilities. However, more animals per study may be required due to limited anatomical space and size of animals. Furthermore, bone metabolism is significantly different with mostly faster bone remodelling. Positive results in rodents may have to be repeated and verified in larger species before human clinical trials can be initiated. Because of the similarities with human bone metabolism the results from sheep carry more authority than those obtained small laboratory animals [34].

The drill hole model as presented here has additional advantages. With this model it is possible to investigate and compare the in vivo characteristics of up to eight different materials in one animal. Also it is possible to compare more than one sample of the same material within an animal but in different locations representing different densities of cancellous bone. This is not just a question of animal protection, but also adds the possibility to compare directly the individual's reaction to various materials. Last but not least, this model allows testing of biomaterials without other than the physiologic mechanical load, which is in contrast to animal models that produced single injection or implantation sites using osteotomy or ostectomies [24,30,49]. These models may not be as suitable for biocompatibility testing of materials in bone as the drill hole model, since it may be difficult to attribute failures to material incompatibility or mechanical problems. Critical sized bone defects in long or cranial bones are the next step once the biocompatibility of the material has been established [31,32,50,51].

Conclusion

The drill hole model in sheep proves to be an excellent animal model to test biocompatibility of biomaterials that should be implanted in bone. Overall, it is a safe model for the experienced surgeon, reduces suffering and numbers of experimental animals and serves as an excellent basis for testing the best materials in a more challenging animal model where mechanical properties play a role such as in critical sized bone defects of long, maxillofacial or cranial bone.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

KN participated in the design of the study, carried out a part of the surgeries, participated in the evaluation of the sections and drafted the manuscript.

JA participated in the design of the study and carried out a part of the surgeries.

AB is responsible for the anatomical part of the study.

BvR participated in the design of the study, carried out a part of the surgeries, supervised the projects and has been involved in drafting and revising the manuscript.

All authors read and approved the final manuscript.

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References

1. Korner D, Palluck E: **Tierexperimentelle Untersuchungen zur Biokompatibilitat von Wurzelfüllmaterialien.** *Dtsch Z Mund Kiefer Gesichtschir* 1991, **15(5):**395-400.
2. Ramakrishna S, Huang Z, Kumar G, Batchelor A, Mayer J: **An introduction to biocomposites.** 1st edition. London: Imperial College Press; 2004.
3. Lane J: **Current approaches to experimental bone grafting.** *J orthop clin north am* 1987, **18(2):**213-225.
4. Hing KA, Best SM, Tanner KE, Revell PA, Bonfield W: **Histomorphological and biomechanical characterization of calcium phosphates in the osseous environment.** *Proc Inst Mech Eng [H]* 1998, **212(6):**437-451.
5. Bauer T: **Bone graft materials. An overview of the basic science.** *J clin orthop* 2000, **371:**10-27.
6. Williams DF: **Consensus and definitions in biomaterials.** In *Advances in biomaterials* 1st edition. Edited by: de Putter C, K dL, de Groot K, Lee A. Amsterdam: Elsevier Science; 1988:11-16.
7. Ratner B: **New ideas in biomaterials science- a path to engineered biomaterials.** *J Biomed Mat Res* 1993, **27:**837-850.
8. Hunziker EB: **Tissue engineering of bone and cartilage. From the preclinical model to the patient.** *Novartis Found Symp* 2003, **249:**70-78. discussion 78–85, 170–174, 239–141
9. Endres S, Landgraff M, Kratz M, Wilke A: **Biokompatibilitätstestung verschiedener Biomaterialien in Abhängigkeit vom Immunstatus.** *Z Orthop Ihre Grenzgeb* 2004, **142(3):**358-365.
10. Ellies LG, Carter JM, Natiella JR, Featherstone JD, Nelson DG: **Quantitative analysis of early in vivo tissue response to synthetic apatite implants.** *J Biomed Mater Res* 1988, **22(2):**137-148.
11. Thull R, Trautner K, Karle EJ: **Modell zur immunologischen Prüfung von Biomaterialien.** *Biomed Tech (Berl)* 1992, **37(7–8):**162-169.

12. Benghuzzi H: **Cytological evaluation of capsular tissue surrounding TCPL implant in adult rats.** *Biomed Sci Instrum* 1996, **32**:81-86.
13. Butler K, Benghuzzi H, Bajpai P, Puckett A, Tucci M, Cason Z, England B: **One year histopathological evaluation of fibrous tissue surrounding TCPL implants using adult rats as a model.** *Biomed Sci Instrum* 1997, **33**:233-239.
14. Held M, Dost P, Erdtmann M, Baumann H, Jahnke K: **Biocompatibility testing of new polymers in a moving implant bed.** *ORL J Otorhinolaryngol Relat Spec* 2000, **62(5)**:247-250.
15. Fini M, Giavaresi G, Torricelli P, Krajewski A, Ravaglioli A, Belmonte MM, Biagini G, Giardino R: **Biocompatibility and osseointegration in osteoporotic bone.** *J Bone Joint Surg Br* 2001, **83(1)**:139-143.
16. Mi FL, Tan YC, Liang HF, Sung HW: **In vivo biocompatibility and degradability of a novel injectable-chitosan-based implant.** *Biomaterials* 2002, **23(1)**:181-191.
17. Fulzele SV, Satturwar PM, Dorle AK: **Study of the biodegradation and in vivo biocompatibility of novel biomaterials.** *Eur J Pharm Sci* 2003, **20(1)**:53-61.
18. Lewandrowski KU, Bondre SP, Wise DL, Trantolo DJ: **Enhanced bioactivity of a poly(propylene fumarate) bone graft substitute by augmentation with nano- hydroxyapatite.** *Biomed Mater Eng* 2003, **13(2)**:115-124.
19. Sousa CJ, Loyola AM, Versiani MA, Biffi JC, Oliveira RP, Pascon EA: **A comparative histological evaluation of the biocompatibility of materials used in apical surgery.** *Int Endod J* 2004, **37(11)**:738-748.
20. Jinno T, Goldberg VM, Davy D, Stevenson S: **Osseointegration of surface-blasted implants made of titanium alloy and cobalt-chromium alloy in a rabbit intramedullary model.** *J Biomed Mater Res* 1998, **42(1)**:20-29.
21. Lopes MA, Santos JD, Monteiro FJ, Ohtsuki C, Osaka A, Kaneko S, Inoue H: **Push- out testing and histological evaluation of glass reinforced hydroxyapatite composites implanted in the tibia of rabbits.** *J Biomed Mater Res* 2001, **54(4)**:463-469.
22. Gao J, Knaack D, Goldberg VM, Caplan AI: **Osteochondral defect repair by demineralized cortical bone matrix.** *Clin Orthop Relat Res* 2004:S62-66.
23. Orsini G, Ricci J, Scarano A, Pecora G, Petrone G, Iezzi G, Piattelli A: **Bone-defect healing with calcium-sulfate particles and cement: an experimental study in rabbit.** *J Biomed Mater Res B Appl Biomater* 2004, **68(2)**:199-208.
24. Patka P, den Otter G, de Groot K, Driessen AA: **Reconstruction of large bone defects with calcium phosphate ceramics – an experimental study.** *Neth J Surg* 1985, **37(2)**:38-44.
25. Choueka J, Charvet JL, Koval KJ, Alexander H, James KS, Hooper KA, Kohn J: **Canine bone response to tyrosine-derived polycarbonates and poly(L-lactic acid).** *J Biomed Mater Res* 1996, **31(1)**:35-41.
26. Pal AK, Pal TK, Mukherjee K, Pal S: **Animal experimentation with tooth derived calcium hydroxyapatite based composites as bone-graft substitute biomaterials.** *Biomed Sci Instrum* 1997, **33**:561-566.
27. Bumgardner JD, Boring JG, Cooper RC Jr, Gao C, Givaruangsawat S, Gilbert JA, Misch CM, Steflik DE: **Preliminary evaluation of a new dental implant design in canine models.** *Implant Dent* 2000, **9(3)**:252-260.
28. Lee TM, Yang CY, Chang E, Tsai RS: **Comparison of plasma-sprayed hydroxyapatite coatings and zirconia-reinforced hydroxyapatite composite coatings: in vivo study.** *J Biomed Mater Res A* 2004, **71(4)**:652-660.
29. Mendes SC, Reis RL, Bovell YP, Cunha AM, van Blitterswijk CA, de Bruijn JD: **Biocompatibility testing of novel starch-based materials with potential application in orthopaedic surgery: a preliminary study.** *Biomaterials* 2001, **22(14)**:2057-2064.
30. Ignatius AA, Betz O, Augat P, Claes LE: **In vivo investigations on composites made of resorbable ceramics and poly(lactide) used as bone graft substitutes.** *J Biomed Mater Res* 2001, **58(6)**:701-709.
31. Kümmerle J: **Experimentelle Studie über die Eignung eines neuen Brushite Calciumphosphatzementes für die kraniofasziale Knochenchirurgie anhand eines ovinen Modells der parietalen Kranioplastik und maxillären Augmentation.** In *Doctoral Thesis* Zürich: Vetsuisse Fakultät, Zürich; 2003.
32. Theiss F, Apelt D, Brand B, Kutter A, Zlinsky K, Bohner M, Matter S, Frei C, Auer J, von Rechenberg B: **Biocompatibility and resorption of a brushite calcium phosphate cement.** *Biomaterials* 2005, **26**:4383-4394.
33. Bush D, England B, Tucci M, Cason Z, Lemos L, Benghuzzi H: **The effect of TCPL devices on tissue-implant interface analysis using adult sheep as a model.** *Biomed Sci Instrum* 1995, **31**:147-152.
34. Nunamaker D: **Experimental models of fracture repair.** *J Clin Orthop Rel Res* 1989:355.
35. Augat P: **Local tissue properties in bone healing: Influence of size and stability of the osteotomy gap.** *J Orthopaed Res* 1998, **16(4)**:475-481.
36. Sarkar M, Patka P, Kinzl L: **First histological observations on the incorporation of a novel calcium phosphate bone substitute material in human cancellous bone.** *J Biomed Mat Res* 2001:329-334.
37. Bosanquet A, Goss A: **The sheep as a model for temporomandibular joint surgery.** *Int J Oral Maxillofac Surg* 1987, **16**:600-603.
38. Arvier J, Scott J, Goss A, Wilson D, Tideman H: **Biological and clinical evaluation of the transmandibular implant.** *Aust Dent J* 1989, **34(6)**:524-529.
39. Rice J, Cowin S, Bowman J: **On the dependence of the elasticity and strength of cancellous bone on apparent density.** *J Biomech* 1988, **21**:155-168.
40. Kuhn J, Goldstein SA, Ciarelli M, Mathews L: **The limitations of canine trabecular bone as a model for human: a biochemical study.** *J Biomech* 1989, **22**:95-107.
41. Kemper M: **Screeningversuch zur Optimierung synthetischer und fibriner Hydrogele als dreidimensionale Trägersubstanz für Bone Morphogenetic Protein, Transforming Growth Factor-beta und Parathormon zur Beschleunigung der Knochenheilung.** In *Doctoral Thesis* Zurich: University of Zurich; 2003.
42. Apelt D, Theiss F, El-Warrak A, Zlinski K, Bettschart-Wolfensberger R, Bohner M, Matter S, Auer J, von Rechenberg B: **In vivo behaviour of three different injectable hydraulic calcium phosphate cements.** *Biomaterials* 2004, **25**:1439-1451.
43. Doernberg Cv: **Evaluation of the biodegradation behaviour of b-tcp cylinders with four different pore diameters.** In *Doctoral Thesis* Zürich: University of Zürich; 2005.
44. Oberle A, Theiss F, Bohner M, Müller J, Kästner SB, Boecken I, Zlinsky K, Wunderlin S, Auer J, von Rechenberg B: **Untersuchungen über den klinischen Einsatz von Brushite- und Hydroxyapatite-Zement beim Schaf.** *Schweiz Arch Tierh* 2005, **147(11)**:482-490.
45. Wolff J: **Über die Wechselbeziehungen zwischen der Form und der Funktion der einzelnen Gebilde des Organismus.** 1st edition. Leipzig: FCW Vogel; 1901.
46. Phemister DB: **The fate of transplanted bone and regenerative power of its various constituents.** *Surg Gynecol Obstet* 1914, **19(3)**:303-333.
47. Urist MR, Dowell TA: **Inductive Substratum for Osteogenesis in Pellets of Particulate Bone Matrix.** *Clin Orthop Relat Res* 1968, **61**:61-78.
48. Urist MR, Silverman BF, Buring K, Dubuc FL, Rosenberg JA: **The bone induction principle.** *Clin Orthop Relat Res* 1967, **53**:243-279.
49. Ignatius AA, Unterricker K, Wenger K, Richter M, Claes L: **A new composite made of polyurethane and glass ceramic in a loaded implant model: A biomechanical and histological analysis.** *J Mater Sci Mater Med* 1997, **8**:753-756.
50. Meinel L, Hofmann S, Karageorgiou V, Kirker-Head C, McCool J, Gronowicz G, Zichner L, Langer R, Vunjak-Novakovic G, Kaplan DL: **The inflammatory responses to silk films in vitro and in vivo.** *Biomaterials* 2005, **26(2)**:147-155.
51. Meinel L, Zoidis E, Zapf J, Hass P, Hottiger MO, Auer J, Schneider R, Luginbuhl V, Bettschart-Wolfensberger R, Rechenberg Bv: **Localized insulin-like growth factor I delivery to enhance new bone formation.** *Bone* 2003, **33**:660-672.

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Anabolic Actions of Parathyroid Hormone on Bone*

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I. Introduction

PHYSICIANS have traditionally considered PTH to be an agent that is catabolic to the skeleton. However, as early as 60 yr ago, Albright and his colleagues (1) and later Selye (2) noted that the peptide extract could also be anabolic to the skeleton, producing increases in bone tissue in animals. The potential for an agent that can increase bone mass and hence reverse the skeletal defect in patients with osteoporosis is great, particularly if in doing so it also repairs microarchitectural damage. The agents currently available in the United States for treatment of osteoporosis, estrogens and salmon calcitonin, primarily stabilize bone mass and prevent further loss of bone, although a transient small increment in mass is often reported, particularly in patients with elevated levels of bone remodeling. This increase is not a true anabolic effect but is related to the temporal effects on turnover in which resorption declines initially followed by a reduction in formation that may take several months. Fluoride, in contrast, does increase bone mass by a true anabolic action, but there is controversy about the quality of the bone formed. Two recent controlled studies failed to find a reduction in fracture recurrence (3, 4), although the dose may have been excessive, and consequently, fluoride is currently not approved for treatment of osteoporosis in the United States. Thus, if PTH is capable of improving bone mass and quality, the peptide will have a potential therapeutic role in osteoporosis. This review summarizes the data available to date, starting at the cellular level.

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II. Target Cells for PTH in Bone

Despite the fact that the major action of PTH in bone is to stimulate osteoclastic bone resorption, a compelling body of evidence has been published over the last two decades indicating that cells of the osteoblast lineage are the principal, but not necessarily the only target (5, 6). A detailed morphological description of a distinct "PTH target cell" (PT cell) was provided recently by Rouleau *et al.* (7, 8). Located among clusters of differentiated osteoblasts, this is a mononucleated cell with a large soma and long cytoplasmic processes extending toward the bone surface and also toward nearby blood vessels. Based on a positive reaction for alkaline phosphatase and the occasional observation of hybrid cells that exhibited ultrastructural features of both the PT cell and differentiated osteoblasts, Rouleau *et al.* (7, 8) concluded that the PTH-responsive cell is indeed of the osteoblast lineage.

The concept that osteogenic cells, rather than the osteoclast, were the primary targets for PTH as well as for other agents that stimulate bone resorption was first formally proposed in a classic paper by Rodan and Martin in 1981 (9). Experimental evidence in support of this hypothesis was obtained when investigators developed successful techniques for studying functionally viable osteoclasts *in vitro*. Thus, Chambers and his colleagues (10) showed that when osteoclasts were physically separated from other bone-derived cells on a devitalized slice of bone, they did not respond to PTH. However, when primary cultures of osteoblasts or osteoblast-like cell lines were added to the culture, PTH then

induced an increase in resorption (11). These functional studies were supported by autoradiographic evidence suggesting a lack of specific PTH binding in osteoclasts (6, 7). Subsequent studies from Chambers' group and others (11–14) suggested the existence of a soluble osteoclast-stimulating factor(s) released by osteoblasts in response to PTH. To date, the identity of this factor has remained elusive, although recent reports have indicated that rather than being soluble, it may be membrane- or matrix-bound (15).

This body of work shifted the emphasis away from the osteoclast as a target cell for PTH despite earlier immunocytochemical evidence for this in fixed bone sections (16). However, in the past 2 yr, evidence has been presented by three independent groups that there are also PTH receptors in osteoclasts. Duong *et al.* (17) reported that bovine PTH(1–34) had an *inhibitory* effect on bone resorption by purified cultures of chick osteoclasts. A stimulatory response was observed, however, in less pure populations with a greater density of contaminant osteoblasts. Moreover, these authors presented autoradiographic evidence for the existence of PTH receptors in both avian and mammalian osteoclasts, postulating that these receptors mediate the observed direct inhibitory effects. Teti *et al.* (18) also described specific binding of radioiodinated PTH to avian osteoclasts, and using a biotinylated PTH binding method amplified by fluorescein isothiocyanate-labeled avidin, Agarwala and Gay (19) demonstrated binding of bovine PTH(1–84) to living chick osteoclasts. In the latter case, several characteristic properties of receptor-mediated hormone binding were described, including saturability, time and temperature dependence, and hormone specificity.

We must, therefore, at present acknowledge the intriguing prospect that both osteoblasts and osteoclasts express functional PTH receptors. This raises the possibility of dual regulatory mechanisms for PTH action on bone with the peptide perhaps exerting a direct inhibitory effect on the osteoclast, and an indirect stimulatory effect mediated by osteoblasts.

III. *In Vitro* Studies on the Anabolic Effects of PTH

A. Organ and cell cultures

While the earliest organ culture studies quickly confirmed PTH's potent bone resorbing activity (20), demonstration of its anabolic activity by this technique has not been so easy. Using Gaillard's original method of culturing embryonic mouse radii, Herrmann-Erlee *et al.* (21) showed that synthetic bovine PTH(1–34) ($\leq 5 \times 10^{-9}$ M) increased osteoblast number and osteoid formation in more than half of the samples studied. However, PTH decreased collagen synthesis in cultured fetal rat calvariae (22), an observation that has been confirmed in neonatal mouse calvariae (23) and, more recently, in fetal rat parietal bones (24), and is attributed to a PTH-induced inhibition of steady-state levels of procollagen messenger RNA (mRNA) (25, 26). This inhibitory effect of PTH on differentiated osteoblasts was corroborated by reductions in alkaline phosphatase activity (27, 28), osteocalcin synthesis, and collagen synthesis in cultured osteoblastic cells (26, 29–31). However, although PTH decreases osteoblastic synthesis and secretion of collagen and other peptides, it appears to stimulate osteoblast replication as measured by the incorporation of [^{3}H]thymidine into DNA in bone organ cultures (32, 33). This observation suggests that the anabolic effect of PTH might be mediated by an increase in the proliferation of osteoblast precursors or osteoprogenitor cells (34), which has been confirmed in some, although not all (35), cell culture models. Low concentrations of PTH stimulate proliferation in rat osteosarcoma-derived osteoblast-like cells (ROS 17/2, UMR 106), rat calvarial osteoblasts (28, 36), and in primary cultures of human bone cells of osteoblast phenotype, and human osteosarcoma cells (34). Interestingly, there is evidence to suggest that the PTH-induced proliferation of osteoblast precursors may be mediated by the osteoblasts themselves in a process that requires cell-to-cell contact (37). In general, the effects of PTH on mitogenesis are associated with a reduction in the expression of the osteoblast phenotype. However, an anabolic effect of PTH on bone that is independent of its mitogenic action should not be discounted. In this regard, Howard *et al.* (38) and, more recently, Canalis and his colleagues (33) have demonstrated that, as is the case *in vivo* (see below), the manner in which PTH is administered *in vitro* profoundly influences its effects on bone formation. Thus, in cultured fetal rat calvariae, continuous treatment with PTH for 24–72 h inhibited type I collagen production as previously reported (22, 26), but transient exposure for 24 h followed by incubation in PTH-free medium resulted in a stimulation of collagen synthesis (33). Furthermore, although [^{3}H]thymidine incorporation was increased in both circumstances, neither the inhibitory nor the stimulatory effects of PTH on collagen synthesis appeared to be dependent on mitogenesis because neither was affected by the presence of the inhibitor of DNA synthesis, hydroxyurea. The stimulatory effect on collagen synthesis was also observed in bones that had been stripped of periosteum, which contains the principal source of osteoblast precursors and is the site of most of the mitogenic effects of PTH. These results imply that PTH can have stimulatory effects on differentiated osteoblasts as well as on the differentiation of preosteoblasts. This is supported by the fact that, contrary to the studies mentioned earlier (27, 28), some osteoblastic cells respond to PTH with an increase in alkaline phosphatase, a time-honored marker of the differentiated osteoblast (39, 40). As a general note, it seems that the response of different osteoblast cultures to PTH is variable and depends crucially on whether they are primary cultures or cell lines, the species and age of the source, and the initial degree of differentiation in culture (41).

B. Role of growth factors in orchestrating the anabolic action of PTH

Recent studies in several laboratories have provided convincing evidence that the PTH-induced stimulation of bone formation is mediated by local growth factors, with insulin-like growth factors (IGF)-I and -II and transforming growth factor-β (TGFβ) being the primary candidates. IGF-I and II

enhance collagen production by two independent methods: stimulation of osteoblast replication and increasing the synthesis of type I procollagen mRNA by differentiated osteoblasts (42). PTH stimulates the release of IGF-I from fetal rat calvariae (33) and of both IGF-I and II from mouse calvariae (43) and also increases the transcript and polypeptide levels of IGF-I in osteoblast-enriched primary cell cultures (44). Moreover, the stimulatory effect of transient PTH administration on type I collagen synthesis in fetal rat calvariae is abolished by IGF-I neutralizing antibodies (33), an effect that is again independent of PTH's mitogenic action, which was unaffected by the antibodies. In addition to increasing osteoblastic synthesis of IGFs and of IGF binding proteins, PTH may also increase the sensitivity of osteoblasts to IGF-I by increasing the affinity and/or the number of IGF receptors. This provides a mechanism for the synergistic effects of PTH and IGF-I on bone formation observed both *in vitro* and *in vivo* (45).

Members of the TGFβ supergene family, which include TGFβ 1 and 2 and bone morphogenetic proteins 2–7 are clearly important bone growth factors (46–48). Indeed, bone is one of the most abundant sources of TGFβ in the body (49). TGFβ is synthesized and secreted by osteoblasts in a biologically inactive, latent form and is incorporated in the extracellular matrix. Osteoblasts not only produce TGFβ but they also possess high affinity receptors for it, providing the opportunity for autocrine stimulation of osteoblast replication (50). Moreover, a recent study suggests that TGFβ can increase the number of functional PTH receptors in osteoblasts (51). TGFβ is a potent stimulator of bone formation both *in vitro* (52, 53) and *in vivo* (54–56). While PTH does not appear to regulate osteoblastic synthesis of TGFβ (44), its initial action to stimulate osteoclastic bone resorption results in the liberation of significant amounts of TGFβ from the matrix. Furthermore, the acidic microenvironment in the subosteoclastic resorption zone appears to be capable of converting TGFβ from latent to active form (57). Once released from bone, the activated TGFβ could presumably stimulate osteoblast chemotaxis, replication, and differentiation in the vicinity of the resorption lacuna and possibly also act to inhibit osteoclast activity and recruitment (46, 58). TGFβ is therefore an ideal candidate for the putative "coupling factor" that tethers bone formation to bone resorption in the remodeling cycle. Other osteoclast-accessible, matrix-bound growth factors that could act in a similar fashion to mediate an anabolic effect of PTH include IGF-I and II (43, 59), platelet derived growth factor (60), and acidic and basic fibroblast growth factors (61, 62), although evidence for involvement in this PTH effect is currently only available for the IGFs. It is worth noting, however, that while this indirect, osteoclast-dependent mechanism of stimulating osteoblastic bone formation may well be operative, it is not essential for eliciting PTH's anabolic action. Tam *et al.* (63) showed that in short-term experiments, intermittent injection of PTH in rats resulted in an increase in bone-forming surfaces and apposition rate without a change in resorption surface and, more recently, Hock *et al.* (64) reported that the anabolic action of PTH in rats was unaffected by treatment with the resorption inhibitors, calcitonin and dichloromethylene bisphosphonate (see below).

C. Second messengers

The fact that PTH can have both catabolic and anabolic effects on the skeleton raises an intriguing question: How does a single hormone achieve such separate and opposing actions in bone, especially as both actions appear to be mediated either directly (formation) or indirectly (resorption) by the same cell type, namely the osteoblast? As mentioned earlier and discussed in more detail below, part of the answer may be the differential response of the target cells depending on whether PTH is delivered continuously, which appears to favor resorption, or in an intermittent fashion which appears to favor formation. An alternative explanation may lie in the ability of PTH to activate at least two different intracellular signal transduction pathways in osteoblasts (64a) (Fig. 1). These are cAMP-dependent protein kinase A (cAMP/PKA) (65, 66), and the calcium/protein kinase C (Ca^2/PKC) pathways (67, 68). At the present time it is not clear which cellular functions in osteoblasts are regulated by which pathway (69). Studies in this area are complicated by the fact that both osteoblasts and osteoclasts "use" the same transduction mechanisms. Thus, stimulation of the cAMP pathway in osteoblasts by experimental agonists, such as forskolin, could theoretically result in stimulation of resorption but this effect may be counterbalanced by simultaneous activation of the cAMP pathway in osteoclasts, which is inhibitory in effect (70, 71). The same problem applies to the Ca^2/PKC pathway, which has recently been shown to be present in osteoclasts (72–74), as well as osteoblasts (67, 68). Perhaps reflecting this difficulty, in two recent publications it has been concluded that the cAMP pathway in osteoblasts is either of *major* (75) or *minor* (76) importance in conveying the stimulatory effect of PTH on bone resorption. Similarly, there is little definitive information on the second messengers responsible for mediating the anabolic action of PTH. Studies with osteoblast-like cell lines indicate that both the cAMP/

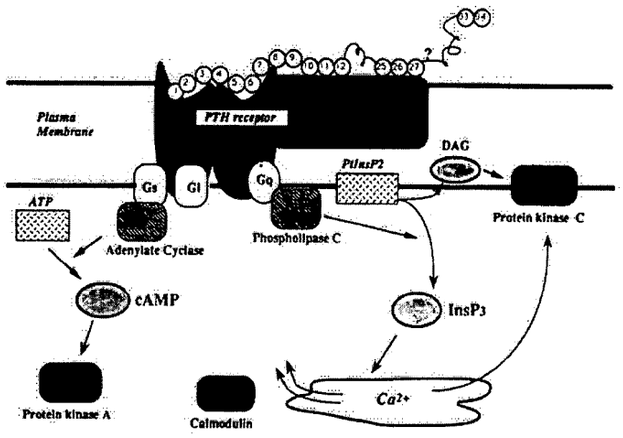

FIG. 1. A model for PTH signal transduction, incorporating both the cAMP/PKA and Ca^{2+}/PKC pathways. [Reproduced with permission from A. Fujimori *et al.*: *Endocrinology* 130:29–36, 1992 (64a). ©The Endocrine Society.]

PKA and the Ca^{2+}/PKC systems play a role in the regulation of proliferation as well as phenotypic expression (75, 77, 78). Based on experiments in which the cAMP and the intracellular Ca^{2+} pathways were selectively activated by different PTH fragments, Lowik *et al.* (79) suggested more than 5 yr ago that osteoblasts contain two distinct receptors with one being coupled to adenylate cyclase and the other involved in the elevation of intracellular Ca^{2+}. However, this hypothesis remains unsubstantiated (69). The precedence for subclasses of receptors conferring specificity for the second messenger pathway exists in other systems. For example, the α-adrenergic receptor is classified into α_1- and α_2-subtypes with the former assigned to the phosphatidylinositol pathway and the latter to the inhibition of adenylate cyclase (80, 81). Modeling of the PTH receptor site in canine kidney (82) suggests the existence of either two separate receptors for PTH(1–84) and PTH(1–34) with similar affinities, or alternatively, a second binding site for PTH(1–84) on the same receptor. Another possibility is that the local conditions in and around osteoblasts could influence the balance between the two pathways. For instance, in rat osteosarcoma cells (ROS 17/2.8) it is known that the presence of retinoic acid influences inositol polyphosphate metabolism, that a decrease in pH activates phospholipase C, and that PTH-stimulated adenylate cyclase activity is enhanced by glucocorticoids (81).

Although it is now firmly established that growth factors play a role in mediating the anabolic effects of PTH, there may be other underlying mechanisms. Finkelman *et al.* (83) recently reported that PTH enhances the proliferation of TE-85 human osteosarcoma cells by a mechanism that does not involve either increased cAMP production or enhanced secretion of IGF-I, IGF-II, or TGFβ. The calcium channel blocker, verapamil, reduced cell growth, both basal and PTH-stimulated, but a significant stimulation was still observed, suggesting that, while a rise in intracellular calcium may be involved in mediating the PTH response in this model, it is not the only second messenger.

While studies on the anabolic action have necessarily focused on osteoblasts and bone formation, we should not overlook the potential that "anticatabolic" effects might also alter the balance between formation and resorption in favor of the former. As mentioned above, Duong *et al.* (17) have reported inhibitory effects on the activity of osteoclasts in osteoblast-deficient cultures. Using the resorption pit assay, we have found evidence for an inhibitory effect on osteoclast function even in the presence of osteoblasts (84). While osteoclasts were activated by PTH and the average number of pits per resorption focus was increased, the median plan area of the individual resorption pits was substantially reduced. Such an effect is consistent with histomorphometric data in patients with primary hyperparathyroidism in which there is increased activation frequency of bone remodeling units but a reduction in the depth of the resorption lacunae (see below). Incidentally, Fenton *et al.* (85) have recently reported that a highly conserved pentapeptide region of PTH-related protein (PTHrp[107–111]) is a potent inhibitor of osteoclastic activity *in vitro*.

In conclusion of this section, these basic studies have elucidated several possible mechanisms for a direct anabolic action of PTH on the skeleton. These include cytokine-mediated stimulation of osteoblastic proliferation and activity and, possibly, inhibition of osteoclast activity (Fig. 2). The remainder of this review will focus on PTH action in intact animals and humans.

IV. Animal Data

The majority of data supporting an anabolic effect of PTH comes from animal studies. Although the rat has been the principal species in which the effects of PTH have been evaluated using a variety of models of bone loss, data are also available for the dog. There is no report of an anabolic action of PTH in any primate species as yet.

A. Early discoveries

Early results were all performed using parathyroid extract (PTE) rather than purified PTH. The earliest report of increased bone density by PTE (8 U sc daily) in rats was that of Bauer and associates (1) but they did not comment on the significance of the finding. Convincing evidence that PTE could stimulate bone formation, in addition to bone resorption, was obtained histologically shortly thereafter by Selye (2) and Pugsley and Selye (86). They observed increased proliferation of bone cells, both osteoclasts and osteoblasts, shortly after a single injection of 20 U PTE to rats. If the treatment was continued, an increase in bone tissue resulted

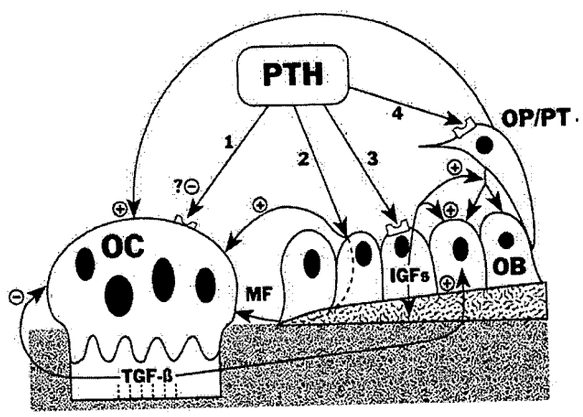

FIG. 2. Diagram illustrating some of the diverse effects of PTH on bone. Stimulation of osteoclastic bone resorption is mediated indirectly via osteoblasts or the putative parathyroid (PT) cell (*pathways 2 and 4*). The signal from osteoblasts/PT cells to osteoclasts may be conveyed by soluble osteoclast-stimulating factor(s) or possibly a membrane or matrix-bound factor (MF). In addition to this indirect communication, there is growing evidence that PTH may act directly on osteoclasts, perhaps exerting an inhibitory influence (*pathway 1*). The anabolic action of intermittent PTH administration is likely mediated by growth factors, including TGFβ, released from the matrix and activated by osteoclasts, and by IGF's I and II, produced by the osteoblasts themselves (*pathway 3*). The growth factors enhance both the replication and activity of osteoblasts. See text for further details. OC, Osteoclast; OB, osteoblast; OP/PT, osteoprogenitor/"parathyroid cell"; MF, membrane- or matrix-bound factor.

and the osteoclast number declined to normal. Interestingly, they noted that treatment with lower doses of PTE (5 U on alternate days) led to proliferation of osteoblasts only. Selye (2) suggested that PTH given in small doses, would lead to a stimulation of osteoblasts and bone apposition without a previous osteoclast formation and fibrous transformation of the bone marrow. Using a similar dose of PTE in rats, Seyle's results on the stimulation of osteoblasts by PTH were confirmed by Jaffe (87) and Burrows (88). Shelling *et al.* (89) directly measured rat femurs and demonstrated an increased ratio of dry femur weight in rats treated with PTE daily for 14 or 21 days. Using a single dose of 1000 U PTE, Heller *et al.* (90) showed a rebound of osteoblast number and activity in 4–7 days after initial inhibition, suggesting a long lasting effect of PTE. Biochemical analysis of matrix materials and radiological density measurements were later added, confirming the anabolic action of PTH in the 1970's (91, 92). Increments in the calcium accretion rate into the skeleton of the order of 45% were noted after chronic administration of PTE in rats (93). A positive calcium balance with daily PTE administration was observed by Cramer *et al.* (94). Using rats on normal and low phosphate diets, the positive calcium balance was observed in 5-day periods after injection of PTE (50 U twice daily for the first 3 days). The early results on the anabolic effect of PTH on animal skeletons have been extensively reviewed previously by Parsons (95).

B. Quantitative determination of the anabolic action of PTH

During the last decade improved analytical techniques and the wide availability of purified PTH fragments led to a renewed interest in the study of the anabolic action of PTH on bone based on sensitive quantitative determinations of the skeleton and its remodeling processes. The apparent success of daily administration of low doses of PTH to humans (96–98) (see below) and in controlled data using dogs (99, 100) further prompted this interest.

In rats, Hefti *et al.* (101) showed an increase in total body calcium, as well as calcium content in 11 different bones, by administering 75 U human PTH (hPTH) (1–34)/day. Tam *et al.* (63) reported an increase in bone formation surface and trabecular bone volume, but not in resorption surface, in rats treated intermittently with 0.4–102.4 U/rat/day of PTH molecules for 12 days. Gunness-Hey and Hock (102), using a novel method of separating cortical and trabecular bone, demonstrated a significant dose-dependent increase in cancellous bone, but not in cortical bone, after PTH injection. This result has been confirmed by several other authors (103–107). The use of histomorphometric methods to quantitate the gain in cancellous bone volume and the changes in bone formation surface or mineral apposition rate have contributed a greater understanding of the mechanism of PTH action (63, 105, 108–114). Results from these experiments showed that the anabolic action of PTH, when given by daily injection, is consistent and proportional to the dose used in the experiment. Injection of PTH for as short a treatment period as 12 days (115) or for as long as 175 days (103) has been shown to effectively increase cancellous bone mass. However, differences in the rapidity and duration of the responses are not known because all information was gathered at the end of the experiments (*i.e.* 12–175 days). Both male and female rats respond to PTH treatment equally well (104, 113) and the age of the rats that respond to PTH can vary from prenatal (92) to 2 yr (113, 116). An interruption in the daily therapy of 4 days results in significant loss of the stimulated bone formation activity, and a consequent loss of bone mass can be observed as early as 8–12 days after the withdrawal of the hormone (111). In all published data the anabolic action of PTH is most evident in cancellous bone while increases in cortical bone mass are either not observed or less pronounced. While the bulk of the data was collected from cancellous enriched regions of tibia or femur, a few studies have observed similar changes in vertebrae (112, 113, 117). The percentage improvement in the mass of various bones, and even in different parts of the same bone, may be related to the different proportions of cortical and trabecular bone and to the metabolic activity in each type of bone. The notion, interpreted from human studies, that cancellous bone is increased by PTH at the expense of cortical bone (96, 98) has not been seen in the rat (63, 118). However, this may be a limitation of the model, since in the cortical bone of rats there is little Haversian remodeling, which may be a prerequisite for such an effect. The dietary calcium intake is also proportionally much higher in the laboratory rat than in humans, making it unnecessary to remove calcium from cortical bone to support cancellous bone formation elsewhere. Unlike the situation suggested for sodium fluoride, the bone formed under stimulation by PTH appears to be of normal quality. The increases in mass are accompanied by an improvement in biomechanical strength of vertebral bodies and this remains even after normalization for both cross-sectional area and bone mass (114, 119). The studies involving treatment with PTH in rats are summarized in Table 1.

C. Preventive and curative treatment of osteopenia with PTH

Early experiments with PTH were performed in intact animals with normal skeletal status. Despite earlier concerns regarding their suitability, several rat models of osteoporosis have been developed, indicating that rats may indeed be useful in the study of the pathogenesis and treatment of osteoporosis in humans. For example, in rats, ovariectomy, orchidectomy, and immobilization of limbs can induce bone loss in the skeleton with increments in bone turnover as is seen in humans. The therapeutic agents used in humans, estrogen, calcitonin, and bisphosphonates (120, 121), reduce bone turnover and prevent further bone loss in the rat models as they do in humans without substantially restoring bone mass or architecture. When tested in various rat models of osteoporosis, intermittent injection of PTH both prevents loss and restores bone mass caused by ovariectomy (103, 104, 108–110, 113, 118), orchidectomy (104), and hemicordectomy (109). The magnitude of the increase (in percentage terms) in bone mass induced by PTH administration in osteoporotic animals is generally greater than that obtained in intact animals. Conventional treatment with antiresorptive agents will not restore lost bone mass or structure. Addition

TABLE 1. Quantification of the anabolic action of PTH on rat bones

Animals	PTH	Dose (μg/kg/day)	Length of Treatment	Oper	Bone	Percentage change in			Ref.
						TBV	ash wt[e] or calcium	BMD	
6 mo ♀ Wis	bPTH 1-34	75[a]	3 wk	Int	Whole body		20		(110)
300 g ♂ SD	bPTH 1-84	0.4–102[a]	12 d	Int	Femur	0–60			(64)
4–6 wk ♂ SD	hPTH 1-34	20–100	12 d	Int	Femur		6–60		(103, 133) (107)
10 wk ♀ SD	hPTH 1-34	6.0	26 wk	Int Ovx	Femur		4 15		(104)
5 wk ♂ SD	hPTH 1-34	80	12 d	Int	Femur		34 (Trab) 12 (Cort)		(105)
				Ochx	Femur		37 (Trab) 19 (Cort)		
5 wk ♀ SD	hPTH 1-34	80	12 d	Int	Femur		18 (Trab) 5 (Cort)		(105)
				Ovx	Femur		14 (Trab) 10 (Cort)		
4 wk ♂ SD	hPTH 1-34	80	12 d	Int	Femur	100	31 (Trab) 20 (Cort)		(106, 108) (112)
8 mo ♀ Wis	hPTH 1-34	60	35 d[b]	PTX +Ovx +Cx	Vert Tibia	70 (Trab) 30 (Cort) 300 (Trab) 12 (Cort)			(110)
3 mo ♀ SD	hPTH 1-34	80, 160	35 d	Ovx	Femur Vert Tibia	70 550	34 49	13 12	(119)
4 mo ♀ SD	hPTH 1-34	1.5	28 d[c]	Ovx	Tibia	46		4	(109, 111)
3 mo ♀ SD	hPTH 1-34	80	20 d[d]	Ovx	Femur Tibia Vert	271 (Trab) 4 (Cort)	50 12	4 5	(114)
70 d ♂ Wis	hPTH 1-34	4–120	30 d	Int	Vert	13–59	0–32		(115)
	hPTH 1-84	10–300	30 d	Int	Vert	11–45	5–24		
3 mo ♂	bPTH 1-34	80	21 d	Int	Vert			9	(114)
23 mo ♂			21 d	Int	Vert			14	(114)
24 mo ♀	hPTH 1-34	80	21 d	Int	Vert			16	(114)
24 mo ♀	hPTH 1-34	160	14 d	Ovx	Femur	178		44	(117)
5 mo ♀	rPTH 1-34	40	28 d	Ovx	Tibia Femur	175 198 165	0 2 9	(109)	

Abbreviations: bPTH, Bovine PTH; wk, week; mo, month; d, day; TBV, trabecular bone volume; BMD, bone mineral density; Vert, lumbar vertebrae; Trab, trabecular bone; Cort, cortical bone; Int, intact; Ovx, ovariectomy; Ochx, orchidectomy; Cx, hemicordectomy; Oper, operation; SD, Sprague Dawley; Wis, Wistar.

[a] Unit of PTH/rat/day.

[b] Treatment begins 11 wk after the operation.

[c] Treatment begins 4 wk after the operation.

[d] Treatment begins 40 days after the operation.

[e] Ash wt. was measured as calcium content in either trabecular or cortical bone of the distal femur.

of an anabolic agent to the antiresorptive treatment should in theory achieve both a reduction in bone resorption and an enhancement of bone formation. This hypothesis is supported by our recent study of the effects of treatment of bone loss in ovariectomized rats with a combination of estrogen and PTH. The results indicated that such combined treatment was not only capable of increasing cancellous bone volume but also of reestablishing trabecular connectivity (108) (Fig. 3). This ability to restore bone mass and structure previously lost (108, 109, 113) has significant implications for the treatment of established osteoporosis in humans, especially for the disease among postmenopausal women, the most common form of the disorder, estimated to affect some 20 million women in the United States.

D. Intermittent vs. continuous administration of PTH

The discrepancy of the findings between PTH injection and severe primary hyperparathyroidism where bone loss was evident (see below) remained a puzzle for a long time (95) until experiments conducted in both dogs and rats suggested convincingly that the difference may, at least in part, relate to differences in the modes of administration of PTH. In dogs, continuous low dose hPTH (1–34) given by infusion (0.05 U/kg/h), mimicking chronic (mild) primary hyperparathyroidism, was associated with increments in osteoid surface and bone resorption at the tissue level, without an increment in bone volume. The authors suggested that this result could be explained by an increase in coupled turnover with equal increments in bone formation and resorption (122). In a separate series of studies, daily sc injection of hPTH (1–34) (15 U/kg/day) was compared with continuous infusion (4.5 U/kg/day) in greyhounds (99, 100). Daily injection of PTH significantly increased indices of bone formation (osteoid surface, plasma alkaline phosphatase, and rate of skeletal accretion of calcium) and resorption (number of osteoclasts and resorption surfaces) and increased trabecular bone volume in the ilium. The daily doses of PTH used in this study were similar to those used in human studies, and the resulting changes in bone parameters were also similar to those in humans (96) and, thus, the findings may be relevant to the human. On the other hand, continuous

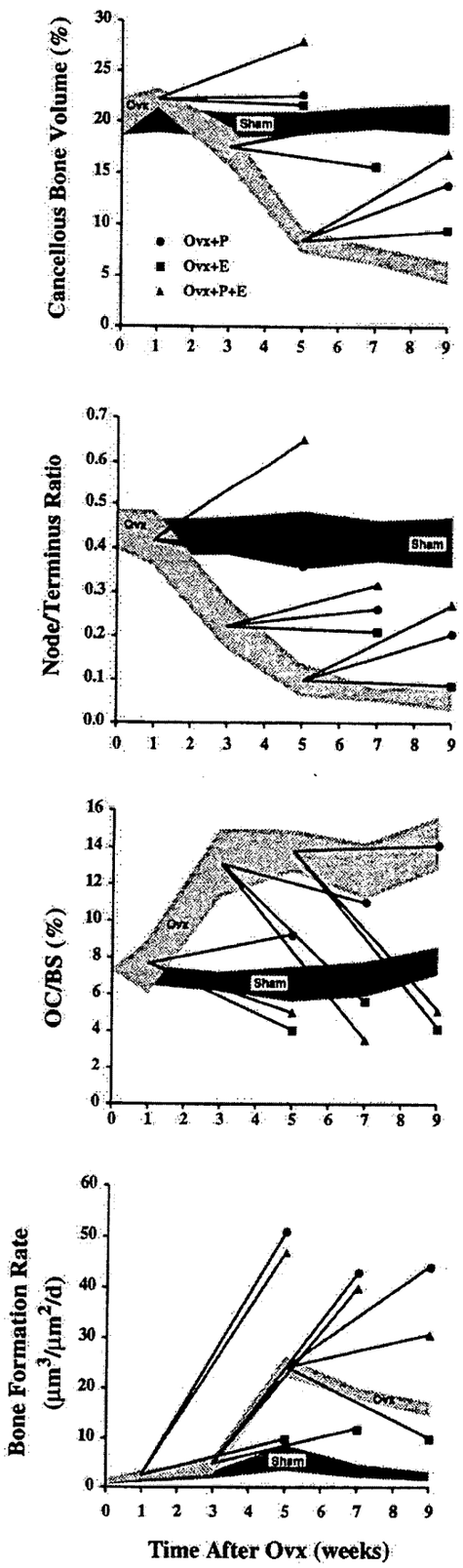

FIG. 3. Effects of treatment of bone loss in ovariectomized (Ovx) rats with a combination of PTH amd estrogen. Five-month-old rats were ovariectomized at time 0. Groups of rats were treated with 40 μg rat PTH(1–34)/kg/day of and/or 30 μg/kg/day of 17β-estradiol for 28 days starting at 1, 3, or 5 weeks after ovariectomy. Cancellous bone volume, node/terminus ratio (an index of trabecular connectivity), osteoclast surface, and bone formation rate were measured in the secondary spongiosa of the proximal tibia after *in vivo* tetracycline labeling. The *dark and light shaded* areas represent the results from the rats that underwent sham operation or ovariectomy, respectively, and were treated only with vehicles. The *circles* represent Ovx animals treated with PTH only, the *squares* represent Ovx animals treated with estradiol only, and the *triangles* represent Ovx animals treated with the combination of PTH and estradiol. Note that even when treatment was delayed for 5 weeks, the combined treatment not only significantly increased cancellous bone volume but also restored trabecular connectivity that had previously been lost. The efficacy of the combined treatment appears to be due to its ability to maintain a high bone formation rate (PTH effect) while decreasing the osteoclast surface (estrogen effect). [Drawn from data presented in Ref. 108.]

infusion of PTH given by Alzet osmotic pumps, using similar doses of PTH, did not significantly increase trabecular bone volume or calcium accretion rate, and only osteoclast surfaces were clearly increased. Tam *et al.* (63) gave rats various doses of bovine PTH (1–84) by either daily injection or continuous infusion. In this experiment, bone formation surfaces were elevated by both methods of administration but resorption increased only if PTH was given by continuous infusion. The net result was an increase in cancellous bone volume when PTH was given by daily injection. Infusion, on the other hand, maintained cancellous bone volume at low doses but caused a decrease in cancellous bone volume when given in high doses. These observations were recently confirmed by Hock and Gera (123) who concluded that both modes of administration increased bone formation in the rat but only intermittent PTH (daily, sc) increased bone mass consistently (123). Thus, the destructive effects of hyperparathyroidism on bone, as reported in early studies of this disease, may be comparable with the high dose experiments while the more recent findings that trabecular bone mass is preserved in mild hyperparathyroidism may be the result of the lower endogenous dose of PTH. This will be discussed in greater detail below.

E. Comparison with skeletal effects of other anabolic agents

Many agents, such as fluoride, several vitamin D metabolites, prostaglandin E_2 (PGE_2), TGFβ, GH, and IGF-I, show the potential to improve bone mass. Although it is generally agreed that fluoride treatment increases cancellous bone volume in chicken, mice, rats, dogs, and pigs as well as in humans, it is difficult to use because of the relatively narrow therapeutic window (124), and the poor quality of bone raises the question, as yet unproven, whether fluoride can ever reduce fracture frequency. Studies using PGE_2 as an agent for new bone production have also yielded very consistent results. PGE_2 appears to activate remodeling and shift bone balance in favor of formation (125, 126) although some *de novo* formation of bone may also occur. This is somewhat different from the apparent effects of intermittent PTH in which uncoupled formation occurs without the need for prior resorption. There are no published data in which a direct comparison of the use of these two agents has been performed. The net effects of long term *in vivo* administration of vitamin D metabolites on bone metabolism can be quite varied. The differences appear to be modulated by the types and dose of vitamin D metabolites used, by dietary calcium intake, and possibly the osteoblast sensitivity, as suggested by Erben *et al.* (127). Studies using TGFβ, GH, and IGF-I as anabolic agents of bone tissue in animals have been scarce (45, 107, 116, 128–130). GH and IGF-I are likely to behave anabolically while TGFβ seems less likely to be an anabolic agent. While GH and IGF-I would be potential candidates for the treatment of osteoporosis, one has to be mindful that these agents have widespread actions on extraskeletal tissues and could be associated with significant undesirable side effects (131). We believe that a better and more efficient approach is to increase the local concentration of growth factors in bone by means of a bone-seeking, growth factor "stimulator," such as PTH.

F. Mechanism of action

Although we do not know the exact mechanism underlying the anabolic action of PTH, there are data that exclude some possibilities. The effects of PTH are not due to compensatory changes in calcitonin secretion because thyroparathyroidectomized rats respond to PTH challenge with an increase in bone mass (91, 109). Hypercalcemia *per se* has no stimulatory effect on bone formation because injection of calcium chloride, instead of PTE, elicits no bone formation response (132). A PTH-induced increase in PGE_2, an anabolic agent in its own right, is unlikely to be the mechanism because coadministration of indomethacin with PTH failed to abolish or modify the anabolic effects of PTH in rats (133). Prior resorption does not appear necessary, as noted above, since stimulation of bone formation without resorptive activity can be seen in rats when PTH is administered by daily injection (63). Furthermore, coadministration of a resorption inhibitor, calcitonin, dichloromethylene bisphosphonate, or estrogen, with PTH fails to block the anabolic response of bone to PTH (105, 108). In fact, as noted above, the combined effects of estrogen administered with PTH are significantly better than either alone, both in terms of bone mass and trabecular structure (Fig. 3 and Ref. 109). The anabolic effects of PTH are not linked to longitudinal growth because aged animals with closed growth plates can still respond to PTH (113) and many studies, with growing animals, showed no specific effect of PTH on long bone length (112, 113, 134).

One of the main functions of PTH is to stimulate the activity of the 1α-hydroxylase in the kidney resulting in an elevation of circulating levels of 1,25-dihydroxyvitamin D :ob1,25$(OH)_2$D] (135). It has been proposed that the increase in 1,25$(OH)_2$D levels may improve intestinal calcium absorption and thus, create a positive calcium balance. 1,25$(OH)_2$D is also known to directly affect the maturation of both osteoblastic and osteoclastic lineage cells (135, 136). It is perhaps not surprising, therefore, that the PTH-induced increment in circulating 1,25$(OH)_2$-D levels has been proposed as necessary for the anabolic action of PTH (113, 118). Indeed, 1,25$(OH)_2$D levels in PTH-treated animals are elevated (108, 113, 118, 137). In one experiment in which rats were given a vitamin D-depleted diet, PTH failed to induce improvement in the bone apposition rate (138) suggesting that increased 1,25$(OH)_2$D may be important for the expression of at least some of the anabolic action of PTH. However, other studies have suggested a less important role for 1,25$(OH)_2$D (106, 108). Supplementation of 1,25$(OH)_2$D to PTH-treated animals does not appear to enhance the anabolic action of PTH (107). Combined treatment of estrogen and PTH produced the greatest improvement in bone mass yet the 1,25$(OH)_2$D level (which is paradoxically reduced by estrogen in rats) (108) was not greater than control levels. This suggests that an absolute elevation of 1,25$(OH)_2$D levels may not be necessary for the increase in bone mass to occur (109).

According to histomorphometric observations, PTH in-

creases bone formation by extending mineralization surfaces and/or the rate of mineral apposition. This is likely the result of increased osteoblast precursor proliferation and/or maturation. As discussed above, there is some indirect *in vitro* evidence as to how this may be achieved. More details of these studies were presented in the last section. This line of reasoning is highly attractive because it has been reported that the anabolic effect of hPTH depends on the presence of GH, a significant positive modulator of IGF-I secretion (107). Further experimentation will be needed to decipher the interaction between PTH, IGF-I, IGF-II, and GH in the anabolic action of PTH in rats.

In concluding this section, intermittent injection of PTH in animals, especially in rats, exhibits a consistent anabolic action on bone tissue. These results suggest that an animal model can provide relevant information on the development of effective PTH treatment regimens for osteoporosis in humans, particularly as PTH administration can apparently restore previously lost trabecular bone mass and structure.

V. Lessons from Primary Hyperparathyroidism: Effects of Endogenous PTH on Bone in Humans

Secondary hyperparathyroidism is known to occur as a homeostatic mechanism in response to decreasing calcium absorption efficiency and decreased 1α hydroxylation of 25-hydroxyvitamin D (138a). This phenomenon has been implicated in age related-bone loss. In addition, primary hyperparathyroidism is often cited as a risk factor for osteoporosis, although in the most recent Consensus Development Conference, this was restricted to severe primary hyperparathyroidism (138b). In fact, recent studies of patients with mild primary hyperparathyroidism suggest that mildly elevated circulating levels of PTH may have beneficial effects, at least on cancellous bone. This section will review these recent findings.

When primary hyperparathyroidism was described 100 yr ago, it was recognized as a syndrome of bone pain, fractures, and renal stones (139–143). The radiologically evident destructive bone lesions and histological observations of severe unbalanced osteoclastic resorption (144–148) led to the belief that high levels of endogenous PTH exerted a catabolic effect on the skeleton. Many years later, increased awareness of this disease (149–152) and the availability of multichannel chemistry screening dramatically changed the mode of presentation of primary hyperparathyroidism. Hence, for the past 15–20 yr, presumably through this phenomenon of early recognition, a more protean milder clinical phenotype is seen most often. Patients are usually asymptomatic and discovered incidentally. Hypercalcemia is mild and clinically evident bone disease is quite uncommon (149, 153–157).

Several studies had suggested that a catabolic effect of PTH on the skeleton might lead to widespread demineralization and fractures even in the presence of mild disease (158–161). In fact, loss of cortical bone has clearly been documented in both severe and mild primary hyperparathyroidism; there is decreased bone mineral density at appendicular sites rich in cortical bone (157, 161–164) and cortices are thinner in iliac bone biopsies (163, 165–167). In contrast, the effect of excess endogenous PTH on cancellous bone is more controversial. While cancellous bone loss at the spine (162, 168) and at the wrist (161) is suggested by some densitometric studies, recent findings indicate conservation of cancellous bone in this disease (157). Similarly, the suggestion that there is an increased vertebral fracture rate in primary hyperparathyroidism (158–161) has not been supported (164, 169, 170). In a recent study by Melton and associates (171), the overall fracture risk was increased in primary hyperparathyroidism compared to age- and sex-matched controls before, but not after, diagnosis. The discrepancies in the data on fracture could be explained, at least partially, by differences in patient populations, nutritional status, disease severity, study designs, and methodology (169). Clearly, the influence of mild primary hyperparathyroidism on fracture risk is an area where further research is needed.

The technique of bone histomorphometry has allowed evaluation of the skeletal effects of the modest elevations of PTH seen in the modern presentation of the disease. While the effects of the disease on bone mass and fracture incidence are still hotly debated, histomorphometric studies performed on different populations over the past 15 yr have failed to show evidence of cancellous bone loss in primary hyperparathyroidism (163, 166, 167, 172–177). Several mechanisms have been proposed to explain the conservation of cancellous bone volume. These include a coupled and balanced increase in bone turnover (176, 178) with increased osteoblastic activity and a prolonged formation period (176), and increased remodeling activation frequency with decreased erosion depth (172, 174). Static variables of bone turnover, such as osteoid surface and eroded surface, are increased in primary hyperparathyroidism suggesting increased bone turnover (173, 175–182). This has been confirmed by dynamic histomorphometric studies performed after skeletal labeling with tetracycline (167, 172, 173, 177, 183, 184). The increased turnover occurs primarily by means of increased activation frequency of new bone remodeling units (174, 183). Moreover, consistent correlations between static and dynamic indices of bone turnover and the mid-molecule of PTH, between these indices and urinary cAMP, and between eroded surface and serum calcium provide strong evidence for a causative role of PTH in the enhanced bone turnover (167).

An anabolic effect of PTH on bone has been suggested by animal studies as well as by clinical therapeutic trials. The observation of osteosclerosis in the metaphyses, skull, vertebrae, pelvis and femora of adults (185–191), and in the metaphyses of children and adolescents uncommonly affected with the disease (192–197) lend credence to this concept. Our recent experience with iliac bone biopsies in primary hyperparathyroidism has provided further evidence that PTH in moderate excess has a protective effect on cancellous bone (157, 167, 197a). The technical developments that have occurred in the field of bone histomorphometry in recent years allow the study of not only mass and turnover, but of bone microarchitecture (198–202). We have

applied this approach to primary hyperparathyroidism to obtain more detailed information on the changes in bone structure. In a study of 27 hyperparathyroid subjects, we observed a striking contrast in the microarchitecture of cortical and cancellous bone between normal individuals and those with mild elevations of endogenous PTH (157, 167, 197a). Cortical thickness was reduced in the hyperparathyroid subjects, confirming previous observations (163, 165) but cancellous bone volume, trabecular number, and importantly trabecular connectivity, as assessed by strut analysis, were higher than in control subjects (Fig. 4). These differences were present in spite of the fact that the ethnic and gender composition of the control group (more young men and more black subjects) might have shifted the difference in favor of a greater bone mass in the control group. Trabecular thickness was not significantly different between patients and controls (167). These findings confirm the higher cancellous bone volume reported by Delling in primary hyperparathyroidism. However, in Delling's study, thicker trabeculae rather than increased trabecular number was the underlying structural component of the increase in bone volume (167, 176). In our study, cancellous bone volume declined with age in both normal and hyperparathyroid subjects. However, while trabecular number decreased and trabecular separation increased with age in the normal subjects, these indices showed no relationship in the hyperparathyroid subjects. Trabecular thickness, in contrast, declined with age in primary hyperparathyroidism but remained unchanged in the normal subjects. The pattern of bone loss that we observed in the normal subjects is consistent with that previously reported (198, 199). It suggests that age-related bone loss, particularly in women, occurs by the osteoclastic removal of entire trabecular plates with those remaining being more widely separated, but generally of normal thickness (198, 199, 203, 204). The pattern of bone loss observed in the hyperparathyroid subjects contrasts with that occurring in normal women after menopause and is more closely related to that described in men (198, 199, 203, 205, 206). We therefore proposed the hypothesis that the effect of increased endogenous PTH was to maintain cancellous bone volume by a mechanism that allows the relative preservation of trabecular architecture. Thus, trabecular plates, although becoming thinner with age, do not perforate and remain connected (167).

That the microarchitecture of cancellous bone is maintained was further demonstrated by comparing normal and hyperparathyroid postmenopausal women matched by age and by number of years postmenopause. A higher cancellous bone volume and an absence of age-related loss of bone structure and trabecular connectivity was again demonstrated in the hyperparathyroid group (207, 208). These studies confirm and extend the observations from Meunier's laboratory (209, 210) and strongly suggest that primary hyperparathyroidism may protect women against the postmenopausal deterioration of cancellous architecture and, possibly, therefore against vertebral fractures. Our findings on the bone structure in primary hyperparathyroidism have been confirmed recently in a separate detailed study of cancellous bone structure and turnover in 69 patients with primary hyperparathyroidism (184). Cancellous bone volume and indices of bone structure did not differ in hyperparathyroid and normal subjects. In spite of the increased cancellous bone remodeling, there was no evidence of accelerated bone loss and no disturbance of cancellous architecture other than a slight thinning of trabeculae, probably resulting from the increased activation frequency. Furthermore, in a postmenopausal subset, contrary to the age-related loss of bone mass and structure observed in normal women, no such loss was noted in the hyperparathyroid group.

Additional evidence for the clinical relevance of these bone biopsy findings is suggested by the following: in our study, bone mineral density (BMD) at the spine, a site rich in cancellous bone, was remarkably preserved, while a moderate deficit in bone mass was observed at the hip, a site of mixed cancellous and cortical composition, with a more substantial decrease at the radius, a site rich in cortical bone (157). These findings are consistent with the selective loss of cortical bone with relative maintenance of cancellous bone volume and structure in primary hyperparathyroidism. The trivial reduction of bone mass in the lumbar spine (~5%)

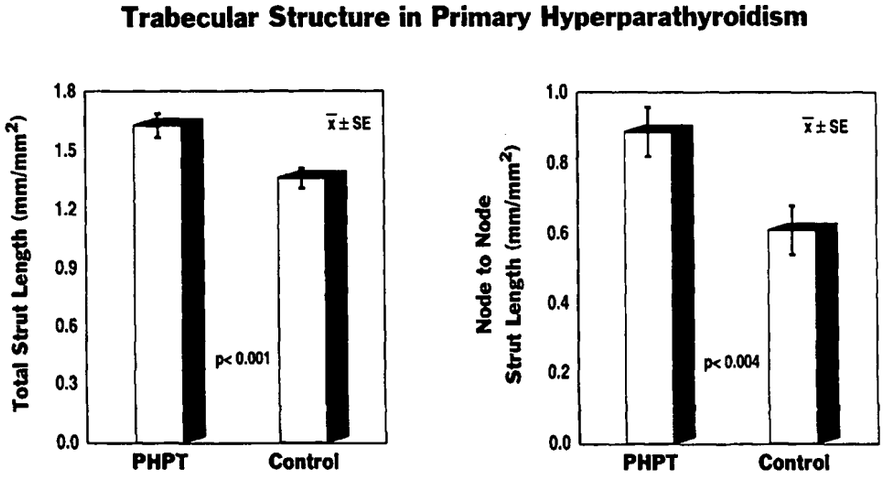

FIG. 4. Indices of trabecular structure in patients with mild primary hyperparathyroidism and controls. The higher total strut length and node to node strut length in the hyperparathyroid patients indicate higher camcellous bone volume and trabecular connectivity, respectively. [Drawn from data presented in Ref. 197a.]

may be the net result of the relative contributions of cancellous and cortical bone when bone mass is measured noninvasively by dual photon absorptiometry (or dual energy x-ray absorptiometry). When cancellous bone was selectively measured at the iliac crest by bone biopsy, a 20% increment in this component was found. Thus, in primary hyperparathyroidism, pure cancellous bone increases while cortical bone decreases, although the possibility that cancellous bone at different skeletal sites responds differently to PTH cannot be ruled out (211). Furthermore, indices of cancellous bone mass and structure (cancellous bone volume, trabecular number, trabecular separation) at the iliac crest were correlated with BMD at the spine, with its higher cancellous component, but not at the hip and wrist at least in women with primary hyperparathyroidism (212, 213).

The cumulative data assembled in our laboratory and by others on bone mass and structure in primary hyperparathyroidism have shown that there is a maintenance or even an increase of cancellous bone volume with a conservation of cancellous microarchitecture and preserved or even increased connectivity of trabecular plates. These findings are observed not only in men and premenopausal women but also in postmenopausal women with primary hyperparathyroidism, at a stage when estrogen deficiency would normally predispose to accelerated bone loss. These data suggest that moderately increased endogenous PTH levels may protect against cancellous bone loss. There is nevertheless a concern that reductions in cortical bone mass consistently observed in this disease may offset the potential utility of PTH as a therapeutic agent in postmenopausal osteoporosis (see below). As we noted above, cortical bone loss does not occur in rats treated with intermittent PTH, and it is not clear whether this is related to the mode of administration, or species differences in response.

VI. PTH Administration in Clinical Studies

A. PTH alone

Despite the animal data (reviewed above) showing, as early as 1929, that PTE and PTH administration could have an anabolic effect on the skeleton, reports of preliminary human trials investigating PTH administration in osteoporotic patients did not appear until the mid 1970s (214, 215). This was, at least in part, because of unavailability of material. Synthetic hPTH (1–34) peptide was not available until 1974, after its synthesis according to the Niall sequence (216) by Tregear and associates (217). Bovine PTH, used therapeutically in a hypoparathyroid patient, had been shown to elicit neutralizing antibodies after short term administration (218). In preliminary data obtained from balance and kinetic studies examining the effects of short-term sc hPTH(1–34) administration, high doses (750 U daily) stimulated resorption in excess of formation, whereas lower doses (450 U) caused an overall improvement in calcium balance, with increases in net intestinal calcium and phosphate absorption, and small increases in bone accretion (215, 219). These early data were therefore comparable to those obtained in both the rat and dog experiments mentioned above.

Combined results of a small multicenter trial were published by Reeve *et al.* (96, 220, 221). A total of 21 osteoporotic patients (16 women and five men) with either vertebral or limb fractures were enrolled in seven centers. The patients and the therapies were somewhat heterogeneous, with one subject receiving pharmacological doses of vitamin D_3 for 2 yr before the study, three taking estrogen before and throughout the trial, and one man receiving testosterone throughout the study. Data were analyzed with each patient serving as his or her own control. Patients had baseline investigations including a radiocalcium kinetic study to allow determination of new bone formation and calculation of bone resorption rate, cortical densitometry of the radius or femur (by single photon absorptiometry), as well as a transiliac crest bone biopsy, with repeat studies after the treatment period.

Patients were treated with once daily sc injections of synthetic hPTH(1–34) in a dose approximately equivalent to 500 U bovine PTH(1–84) for periods of 6–24 months. No serious side effects of the treatment were noted, with only transient hypercalcemia in four cases. Overall, not surprisingly considering the heterogeneity of the group, there was a large interindividual variation in responsiveness to the treatment protocol. Mean serum total alkaline phosphatase increased 15% above baseline in association with increases in urinary hydroxyproline, calcium, and phosphate excretion. Striking increases in new bone accretion measured using a ^{47}Ca-kinetic method were noted (mean increment 144%) with lesser increments in resorption rate (220). Cortical bone density in the group as a whole did not change; however, in nine of 10 paired biopsies on which quantitative analysis could be performed, increases in cancellous bone volume (Cn-BV/TV) were seen (mean increase 92%), as well as increments in osteoid covered cancellous surfaces (mean increase 50%). The one patient who showed no increment in Cn-BV/TV was subsequently found to have developed circulating antibodies to the hPTH peptide. A close correlation was found between the ^{47}Ca accretion rate and Cn-BV/TV at the end of the treatment period.

In contrast, net intestinal calcium absorption did not increase consistently and, more importantly, overall calcium balance did not change positively. The former finding might have been due to a lack of increase in $1,25(OH)_2D$, assuming that any increment in calcium absorption would be the result of improved active transport requiring increased $1,25(OH)_2D$. This thesis was consistent with studies of the hypocalcemic induction of endogenous secondary hyperparathyroidism in which sustained increases in PTH (12 h) appeared to be necessary to stimulate increases in $1,25(OH)_2D$ (222). In two normal subjects in which the kinetics of hPTH(1–34) were studied after injection, rapid clearance from the circulation was observed with return to baseline levels after 6 h (by RIA) and after 40 min using a cytochemical assay (223). Thus, in order to augment intestinal calcium absorption, subsequent studies were performed with the addition of small daily doses of $1,25(OH)_2D$. The latter finding, *i.e.* the lack of positive calcium balance in association with promi-

nent increments in Cn-BV/TV, led to the thesis that exogenous PTH administration in this regimen increased cancellous bone mass at the expense of cortical bone loss, a result shown in a subgroup of patients in whom bone mass had been measured in the femur (98). In order to guard against this potential problem, investigators sought to use hPTH in combination with an antiresorptive agent. In fact, the two patients who had the best response to PTH were those also on estrogen therapy (224).

B. Combination of hPTH with 1,25(OH)$_2$D

To address concern regarding lack of consistent improvement in calcium absorption, Slovik and co-workers (225, 226) added daily oral 1,25(OH)$_2$D (0.25 μg-0.5 μg) to a regimen of daily sc hPTH(1–34) (400–500 U) in eight men with idiopathic osteoporosis (mean age 50 yr) and six women with postmenopausal osteoporosis for 8–24 months. An additional 10 women received alternating 6-week cycles of hPTH(1–34) and 1,25(OH)$_2$D. There were no differences in the results between this latter group and those who received both hormones daily. A similar group of osteoporotic women treated with calcium or 1,25(OH)$_2$D alone served as controls. In four of four male subjects in whom vertebral density measurements were available, trabecular bone density (by single energy computed x-ray tomography) increased on average 98% after 12 months of therapy (Fig. 5). Increments in vertebral trabecular bone density also occurred in the women, but were not as high as those observed in the men (226). Radius density (single photon absorptiometry) did not change. In contrast, in those women treated with calcium or 1,25(OH)$_2$D alone, cortical bone density in the radius decreased. Calcium and phosphorus balance improved in four of four male patients associated with increments in dietary calcium and phosphorus absorption. The conclusion from these studies was that the combination of hPTH(1–34) and 1,25(OH)$_2$D had greater anabolic effects on bone than either agent alone in osteoporotic men and women. Given that cortical bone density did not decrease and that calcium balance increased, the authors concluded that this regimen resulted in a true increase in total bone mass without sacrificing cortical bone.

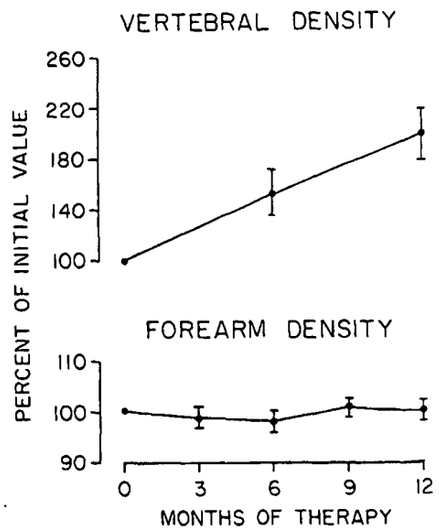

FIG. 5. Changes in trabecular bone density of lumbar vertebrae (*top*) and of cortical bone density of the radius (*bottom*) in osteoporotic men treated with daily sc injection of 400–500 U hTPH(1–34) plus daily ingestion of 15–30 mmol calcium and 0.25–5.0 μg 1,25(OH)$_2$D$_3$. [Reproduced with permission from D. M. Slovik *et al.*: *J Bone Miner Res* 1:377–381, 1986 (225).]

More recently, Neer *et al.* (227) published a controlled trial of 30 osteoporotic women, half of whom received 400–500 U hPTH (1–34) plus 0.25 μcg calcitriol/day while the control group received only calcium supplementation. Total spinal bone mineral density of PTH-treated women by dual photon absorptiometry (DPA) increased 12% over 1–2 yr with no change in the control group. In an unspecified cortical site, BMD decreased by 5.7% in the PTH-treated group *vs.* only 1.7% in the control group. Furthermore, these investigators noted that after 6–12 months, spinal bone mass stopped increasing for reasons that are unclear. There are currently no long term data on the effects of PTH treatment (>24 months). However, data from our group on the effects of PTH over an 18-month period in estrogen-treated women show a progressive increase in spinal bone mineral density without evidence of any plateau effect and without any detrimental effects on cortical bone (228).

C. Combination of hPTH with antiresorptive therapies

More recent investigations of the anabolic effect of hPTH in humans have been performed with combinations of hPTH and antiresorptive therapies. None of these studies has been well controlled or randomized, however. Modern densitometric techniques have allowed further elucidation of the effects of these regimens on different regions of the skeleton.

Hesch *et al.* (229) investigated eight osteoporotic patients treated with hPTH(1–38) instead of hPTH(1–34) as early clinical observations had suggested that the former peptide had somewhat greater potency (230). The protocol was designed to take advantage of PTH as both an activator of skeletal remodeling (see section on ADFR below) as well as an anabolic agent. 720–750 U hPTH(1–38) were given for 8 weeks, with calcitonin administered (in addition to PTH) intranasally (200 U/day) during weeks 2–4 and 6–8. Calcitonin was given alone during weeks 8–10 and no treatment was given during weeks 11–14. The entire cycle was repeated four times over a 14-month period. Density of the spine (assessed by quantitative computed tomography) increased in all subjects (12–89%). Five 'control patients' (three on no therapy, one on sodium fluoride, and one on estrogen) had no increments in spinal BMD. Forearm BMD by single photon absorptiometry (SPA) did not change in treated patients or 'controls.' Some, but not all, patients lost bone after 12 months without therapy. Overall, the combination of PTH and calcitonin appeared to be a somewhat effective anabolic regimen for the skeleton, more effective than calcitonin alone according to the results culled from the literature, but not clearly more effective than hPTH alone. Since neither hPTH nor calcitonin alone was given in the same study, these direct comparisons cannot be made.

Reeve *et al.* (97) studied 24 patients with osteoporosis (vertebral fractures) in an open, nonrandomized 1-yr protocol (97). Twelve subjects received hPTH(1–34), and estrogen for 8 of 12 months (in nine patients) or nandrolone (25 mg every 3 weeks (in three patients). One of these patients stopped her estrogen therapy. The other 12 subjects were treated with sodium fluoride and some of this latter group included patients previously treated with parathyroid peptide. BMD of composite bone (cancellous + cortical) by DPA increased in the fluoride group approximately 13%. In the PTH + estrogen group, spinal BMD increased similarly by both quantitative computed tomography (QCT) and DPA with no change in radius BMD (by QCT) implying that the BMD increment was most likely in cancellous bone (estimated increment 50%), and that there was no cortical loss. A lack of cortical bone loss was also supported by radial bone density which was unchanged in both the PTH and the fluoride groups. This finding was similar to the lack of radial density changes found in the initial multicenter trial when hPTH(1–34) was given alone (without estrogen) but contrasted with findings in the femoral shaft where bone loss was found in a subset of patients (98).

Further investigations were performed on the patients above in the hPTH + estrogen group (231–233). Radioisotopic measures of bone formation showed marked increments (231). Calcium balance improved in most patients, with the mean increment approaching statistical significance. One patient showed a marginal increase in serum binding to hPTH(1–34) of questionable clinical significance. The combination of hPTH(1–34) and estrogen increased the width of new cancellous bone packets as well as the width of trabecular plates and there was a 20% reduction (nonsignificant) in trabecular separation (232) (Fig. 6).

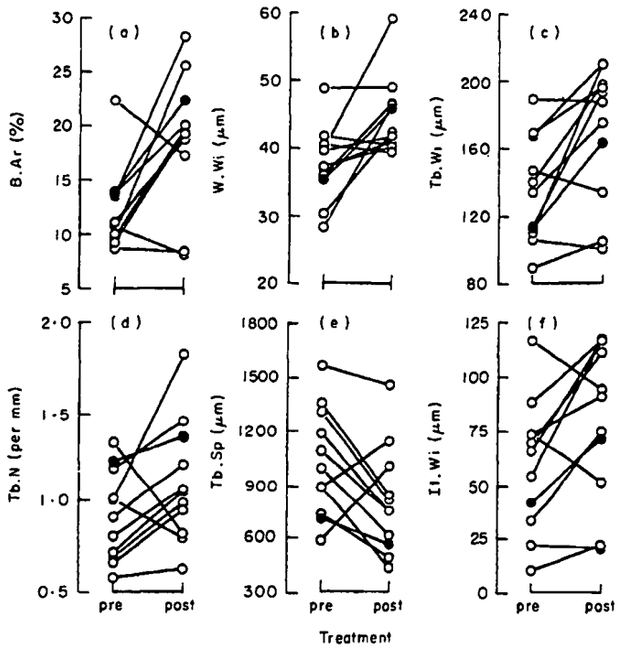

FIG. 6. Changes in trabecular bone structure in iliac crest biopsies of 11 osteoporotic women treated for 12–14 months with a combination of 460–740 "house" units of hPTH(1–34) and hormone replacement therapy (conjugated equine estrogens + norgestrel) for 12–14 months. The patient represented by *black circles* discontinued hormone replacement therapy. B.Ar, Cancellous bone area; W.Wi, wall width of trabecular bone packets; Tb.Wi, trabecular width; Tb.N, trabecular number; Tb.Sp, trabecular separation; It.Wi, interstitial width. The increments in B.Ar, W.Wi, and Tb.Wi were statistically significant ($P < 0.05$–0.01) for the group. [Reproduced with permission from J. N. Bradbeer *et al.*: *Clin Endocrinol (Oxf)* 37:282–289, 1992 (232). ©Blackwell Scientific Publications.]

D. Cyclical (ADFR) therapy

Another potential use of hPTH that must be distinguished from its daily administration to achieve a direct anabolic effect on the skeleton is its use as a skeletal activator in so-called ADFR protocols (A: activate remodeling, D: depress resorption, F: free formation, R: repeat). While both the daily use as well as the sequential use probably activate bone turnover, in the former application the peptide is administered over a long period with the hope of stimulating formation more than resorption. In the latter application, PTH is administered only for a short period to activate a cohort of remodeling units which then allows resorption to be selectively targeted by inhibiting agents. There is evidence to suggest that even intermittent injections of PTH stimulate markers of bone resorption (228, 234). Based on animal data and the observations in primary hyperparathyroidism both discussed above, however, we hypothesize that the resorption cavities formed under the influence of PTH may be shallower and not of the perforative type. Since PTH is not intended to act as an anabolic agent in the ADFR concept, such use is not really within the scope of this review. For completeness, however, the few studies that have used hPTH in ADFR protocols will be discussed.

Twelve osteoporotic patients were treated with hPTH(1–34), 1500 U/day (on the basis of studies in the greyhound dog), for 1 week followed by 3 weeks treatment with 0.25 μg $1,25(OH)_2D$ (235, 236). This cycle was then repeated 15 times. One patient developed a high titer of binding antibodies to the peptide and was thus excluded from the analysis. Mean calcium balance increased, and although individual patients had increments in trabecular bone density, there was no mean change in the group as a whole. Moreover, paired iliac bone biopsies showed no increases in Cn-BV/TV, and osteoid surface and volume and resorption surfaces all decreased. This regimen was clearly a failure, therefore, with the cyclical stimulus resulting in a paradoxical decrease rather than an increment in skeletal turnover. Possible explanations included tachyphylaxis to the activation stimulus or provocation of excess endogenous calcitonin.

Hesch *et al.* studied a heterogeneous osteoporotic group (six women, two men), three having had no previous treatment, and five having previously been treated with sodium fluoride, calcitonin, vitamin D, methenolone (an anabolic steroid), or combinations of these agents before enrollment (237). Patients received 400 U PTH(1–38) for 2 weeks. PTH(1–38) was again chosen because of its presumed greater potency (231). During the second week Etidronate (5 mg/kg)

was also supplied for a total of 2 weeks. There was a subsequent drug-free period of 12 weeks, with the cycle repeated six times. Effects on calcium balance were highly variable, while biochemical indices reflected stimulation of bone turnover. Histological evaluation before and after treatment showed no increase in bone mass.

Another study in 20 osteoporotic patients (17 women, three men) utilized 400 IU hPTH(1–38) daily for 2 weeks (after a 24-h infusion of hPTH) as a skeletal activator followed by 8 weeks of sc salmon calcitonin (20 U/day for 1 week, 50 U/day for 1 week, and 100 U/day for 6 weeks) in half of the patients and no further treatment in the other half (238). Both groups were then given no medication for 30 days (free period) and the cycle was repeated once. $1,25(OH)_2D$ increased above baseline during the 14-day PTH treatment period. This increase was correlated with an increment in calcium absorption (by ^{45}Ca absorption test) from 24% to 30%. Serum alkaline phosphatase and osteocalcin were elevated by the end of PTH administration and remained so after an observation period of 100 days (one cycle). Smaller increases in alkaline phosphatase were seen in those patients given calcitonin. Urinary hydroxyproline was elevated only after the hPTH infusion but not during subsequent sc hPTH administration. Since biochemical changes during the calcitonin phase were so insignificant, it was felt that the PTH activation stimulus was given in too low a dose or too short a duration. The results suggested that PTH might have only modestly stimulated skeletal remodeling with persistent and more significant increases in bone formation over resorption. Bone density findings on these patients were reported in a later paper (239). After 200 days of therapy, in the PTH-treated group, vertebral bone mineral content (by DPA) increased by 13% whereas forearm BMC (by SPA) decreased by 11%. In the PTH + CT group there were no changes in bone density at either site. Histomorphometric analysis of paired biopsies performed before treatment and after two cycles revealed a significant increase in remodeling activation frequency, but no changes in Cn-BV/TV or in the thickness of trabecular bone packets.

VII. Conclusion

In conclusion, the results from human clinical trials on hPTH administration summarized in Table 2 are very prelim-

TABLE 2. Clinical studies employing PTH in the treatment of osteoporosis

Patients sex/ age range	Duration of treatment	PTH fragment dose/regimen	Coadministered agents	Results	Ref.
16♀ 49–78 5♂ 52–61	6–24 mo	Variable but mostly 400–500 U hPTH(1-34)	None	No change in calcium balance 70% increase of cancellous bone volume in iliac crest, bone loss in the distal femur	(96) (214) (215) (220) (221) (98)
8♂ 37–62	1 yr	hPTH(1-34) 400–500 U/day	0.25 μg/day $1,25(OH)_2D_3$ 600–1200 mg/day calcium	98% increase in vertebral trabecular BMD (QCT). No change in radius mid shaft BMD (SPA). Calcium retention improved by 120 mg/day	(225)
15♀	1–2 yr	hPTH(1-34) 400–500 U/day	0.25 μg/day $1,25(OH)_2D_3$ 1000–2000 mg/day calcium	32% ↑ in vertebral trabecular BMD (QCT) 12% ↑ in spinal BMD (DPA) 5.7% ↓ in cortical bone	
15♀	1–2 yr	Control	1000–2000 mg/day calcium	1.7% ↓ in cortical bone No changes in vertebral BMD	(227)
11♀/1♂ Female Mean 64 Male 67	1 yr	hPTH(1-34) 500 U/day	9F given estrogen 5–12 months 2 F/1 M given nandrolone 25 mg/3 wk	Calcium retention improved by 64 mg/day Trabecular vertebral bone density ↑ (QCT) by 50% Cancellous bone volume in iliac crest ↑ by 42%	(232) (97) (231) (233)
6♂/2♀ 33–67	14 mo	hPTH(1-38) 720–750 U/day 8 wk/13 wk cycle total of four cycles	6/13 wk intermittent nasal calcitonin 200 U/day	Vertebral BMD (QCT) ↑ by 12–89% (mean ~15%) No change in forearm BMD (SPA)	(229)

M, Male; F, female; mo, month.

inary but do offer some hope that this agent alone or in some combination with other agents may yield a meaningful anabolic effect on the skeleton with the potential to diminish fracture frequency in patients suffering from osteoporosis. Many questions remain after the investigations reviewed above. These include: 1) To accomplish an anabolic effect of hPTH, is the addition of $1,25(OH)_2D$ required? Does PTH administered as a once daily sc injection increase $1,25(OH)_2D$ by itself and is this associated with increments in gastrointestinal calcium absorption? 2) Will hPTH administered in a dose that will not cause hypercalcemia, alone or in combination with antiresorptive drugs, cause an increase in bone mass when studied appropriately in a randomized, controlled trial? Is the combination of hPTH with antiresorptive therapy more effective than PTH alone? 3) Can bone mass in predominantly cancellous parts of the skeleton be increased without compromising cortical bone sites? 4) What is the incidence of induction of binding antibody to the hPTH amino-terminal peptide? 5) Is there any difference in efficacy between different lengths of the peptide, specifically (1–34) *vs.* (1–38)? 6) Can PTH actually help reverse the loss of trabecular connectivity seen in postmenopausal osteoporosis in addition to increasing trabecular bone volume? 7) If bone density and connectivity improve with hPTH administration, will these effects be maintained when the treatment is discontinued? 8) Finally, if changes in bone mass and structure are positive, will this translate into a reduction in fracture frequency? Much further study is needed to answer these questions.

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References

1. **Bauer W, Aub JC, Albright F** 1929 Studies of calcium phosphorus metabolism: study of bone trabeculae as ready available reserve supply of calcium. J Exp Med 49:145–162
2. **Selye H** 1932 On the stimulation of new bone-formation with parathyroid extract, irradiated ergosterol. Endocrinology 16:547–558
3. **Riggs BL, Hodgson SF, O'Fallon WM, Chao EW, Wahner HW, Muhs JM, Cedel SL, Melton III LJ** 1990 Effect of fluoride treatment on the fracture rate in postmenopausal women with osteoporosis. N Engl J Med 322:802–809
4. **Kleerekoper M, Peterson EL, Nelson DA, Phillips E, Schork MA, Tilley BC, Parfitt AM** 1991 A randomized trial of sodium fluoride as a treatment for postmenopausal osteoporosis. Osteo Int 1:155–161
5. **Neuman WF, Neuman MF, Sammon PJ, Casarett GW** 1975 The metabolism of labeled parathyroid hormone. Calcif Tissue Res 18:263–270
6. **Rouleau MF, Warshawsky H, Goltzman D** 1986 Parathyroid hormone binding *in vivo* to renal, hepatic, skeletal tissues of the rat using a radioautographic approach. Endocrinology 118:919–931
7. **Rouleau MF, Mitchell J, Goltzman D** 1988 *In vivo* distribution of parathyroid hormone receptors in bone: evidence that a predominant osseous target cell is not the mature osteoblast. Endocrinology 123:187–191
8. **Rouleau MF, Mitchell J, Goltzman D** 1990 Characterization of the major parathyroid hormone target cell in the endosteal metaphysis of rat long bones. J Bone Miner Res 5:1043–1053
9. **Rodan GA, Martin TJ** 1981 Role of osteoblasts in hormonal control of bone resorption: a hypothesis. Calcif Tissue Int 33:349–351
10. **Chambers TJ, McSheehy PMJ, Thomson BM, Fuller K** 1985 The effect of calcium-regulating hormones, prostaglandins on bone resorption by osteoclasts disaggregated from neonatal rabbit bones. Endocrinology 116:234–239
11. **McSheehy PHJ, Chambers TJ** 1986 Osteoblastic cells mediate osteoclastic responsiveness to parathyroid hormone. Endocrinology 118:824–828
12. **Perry HM, Skogen W, Chappel JC, Wilner GD, Kahn AJ, Teitelbaum S** 1987 Conditioned medium from osteoblast-like cells mediates parathyroid hormone induced bone resorption. Calcif Tissue Int 40:298–300
13. **Perry HM, Skogen W, Chappel J, Kahn AJ** 1989 Partial characterization of a parathyroid hormone-stimulated resorption factor(s) from osteoblast-like cells. Endocrinology 125:2075–2082
14. **Morris CA, Mitnick ME, Weir EC, Horowitz M, Kreider BL** 1990 The parathyroid hormone-related protein stimulates human osteoblast-like cells to secrete a 9000-dalton bone-resorbing protein. Endocrinology 126:1783–1785
15. **Fuller K, Gallagher AC, Chambers TJ** 1991 Osteoclast resorption-stimulating activity is associated with the osteoblast surface and/or the extracellular matrix. Biochem Biophys Res Commun 181:67–73
16. **Rao LG, Murray TM, Heersche JNM** 1983 Immunohistochemical demonstration of parathyroid hormone binding to specific cell types in fixed rat bone tissue. Endocrinology 113:805–810
17. **Duong LT, Grasser W, DeHaven PA, Sato M** 1990 Parathyroid hormone receptors identified on avian and rat osteoclasts. J Bone Miner Res 5 [Suppl. 2]:s203 (Abstract)
18. **Teti LT, Rizzoli R, Zambonin ZA** 1991 Parathyroid hormone binding to cultured avian osteoclasts. Biochem Biophys Res Commun 174:1217–1222
19. **Agarwala N, Gay CV** 1992 Specific binding of parathyroid hormone to living osteoclasts. J Bone Miner Res 7:531–539
20. **Reynolds JJ** 1972 Skeletal Tissue; in Bourne GH (ed): In: The Biochemistry and Physiology of Bone. Academic Press, New York, pp 70–126
21. **Herrmann-Erlee MP, Heersche JN, Hekkelman JW, Gaillard PJ, Tregear GW, Parsons JA, Potts Jr JT** 1976 Effects of bone *in vitro* of bovine parathyroid hormone, synthetic fragments representing residues 1–34, 2–34, 3–34. Endocrinol Res Commun 3:21–35
22. **Dietrich JW, Canalis EM, Maina DM, Raisz LG** 1976 Hormonal control of bone collagen synthesis *in vitro*. Effects of parathyroid hormone, calcitonin. Endocrinology 98:943–949
23. **Hefley TJ, Krieger NS, Stern PH** 1986 Simultaneous measurement of bone resorption, collagen. Ann Biochem 153:166–171
24. **Vargas SJ, Raisz LG** 1990 Simultaneous assessment of bone resorption and formation in cultures of 22-day fetal rat parietal bones: effects of parathyroid hormone and prostaglandin E_2. Bone 11:61–65
25. **Kream BE, Rowe RW, Gworek SC, Raisz LG** 1980 Parathyroid hormone alters collagen synthesis and procollagen mRNA levels in fetal rat calvariae. Proc Natl Acad Sci USA 77:5654–5658
26. **Partridge NC, Dickson CA, Kopp K, Teitlebaum SL, Crouch EC, Kahn AJ** 1989 Parathyroid hormone inhibits collagen synthesis at both ribonucleic acid and protein levels in rat osteogenic sarcoma cells. Mol Endocrinol 3:232–239
27. **Wong GL, Luben RA, Cohn DV** 1977 1,25-Dihydroxycholecalciferol and parahormone: effects on isolated osteoclast-like and osteoblast-like cells. Science 197:663–665
28. **Majeska RJ, Rodan GA** 1981 Low concentrations of parathyroid hormone enhance growth of cloned osteoblast-like cells *in vitro*. Calcif Tissue Int 33:323–000
29. **Kream BE, Rowe D, Smith MD, Maher V, Majeska R** 1986 Hormonal regulation of collagen production in a clonal rat osteosarcoma cell line. Endocrinology 119:1922–1928
30. **Beresford JN, Gallagher JA, Poser JW, Russell RGG** 1984 Synthesis of osteocalcin by human bone cells *in vitro*. Effects of 1,25 $(OH)_2D_3$, PTH and glucocorticoids. Metab Bone Dis Rel Res 5:229–234
31. **Bringhurst FR, Potts Jr JT** 1981 Bone collagen synthesis *in vitro*: structure activity relations among parathyroid hormone fragments and analogs. Endocrinology 108:103–108

32. **DeBartolo TF, Pegg LE, Shasserre C, Hahn TJ** 1982 Comparison of parathyroid hormone and calcium inonopore A23187: effects on bone resorption and nucleic acid synthesis in cultured fetal rat bone. Calcif Tissue Int 34:495–500
33. **Canalis E, Centrella M, Burch W, McCarthy TL** 1989 Insulin-like growth factor I mediates selective anabolic effects of parathyroid hormone in bone cultures. J Clin Invest 83:60–65
34. **MacDonald BR, Gallagher JA, Russell RGG** 1986 Parathyroid hormone stimulates the proliferation of cells derived from human bone. Endocrinology 118:2445–2449
35. **Centrella M, McCarthy TL, Canalis E** 1988 Parathyroid hormone modulates transforming growth factor β activity, binding in osteoblast-enriched cell cultures form fetal rat parietal bone. Proc Natl Acad Sci USA 85:5889–5893
36. **Partridge NC, Martin TJ,** Studies on the effects of parathyroid hormone on growth of UMR 106 osteogenic sarcoma cells. American Society for Bone and Mineral Research Annual Meeting, 1984 (abstract): A19
37. **Van der Plas A, Nijweide PJ** 1988 Cell-cell interactions in the osteogenic compartment of bone. Bone 9:107–111
38. **Howard GA, Bottemiller BL, Turner RT, Raider JI, Baylink DJ** 1981 Parathyroid hormone stimulates bone formation, resorption oxygen culture: evidence for a coupling mechanism. Proc Natl Acad Sci USA 78:3204–3208
39. **McPartlin J, Skrabanek D, Powell D** 1984 Bone alkaline phosphatase: quantitative cytochemical characterization and response to parathyrin *in vitro*. Biochem Soc Trans 123:894–898
40. **Puzas JE, Brand JS** 1985 Bone cell phosphotyrosine phosphatase: characterization and regulation by calcitropic hormones. Endocrinology 116:2463–2468
41. **Nijweide PJ, Burger EH, Feyen JHM** 1986 Cells of bone: proliferation, differentiation, and hormonal regulation. Physiol Rev 66:855–886
42. **McCarthy TL, Centrella M, Canalis E** 1989 Regulatory effects of insulin-like growth factors I and II on bone collagen synthesis in rat calvarial cultures. Endocrinology 124:301–309
43. **Linkhart TA, Mohan S** 1989 Parathyroid hormone stimulates release of insulin-like growth factor-I (IGF-I), IGF-II from neonatal mouse calvaria in organ culture. Endocrinology 125:1484–1491
44. **McCarthy TL, Centrella M, Canalis E** 1989 Parathyroid hormone enhances the transcript and polypeptide levels of insulin-like growth factor I in osteoblast enriched cultures form fetal rat bone. Endocrinology 124:1247–1253
45. **Howard GA, Spencer EM** 1991 IGF-parathyroid hormone interactions on bone *in vitro* and *in vivo*. In: Spencer E (ed) Modern Concepts of Insulin-Like Growth Factors. Elsevier, New York pp 155–168
46. **Bonewald LF, Mundy GR** 1990 Role of transforming growth factor-beta in bone remodeling. Clin Orthop 250:261–276
47. **Martin TJ, Ng KW, Suda T** 1989 Bone cell physiology. Endocrinol Metab Clin N Am 18:833–858
48. **Centrella M, McCarthy TL, Canalis E** 1991 Transforming growth-factor-beta and remodeling of bone. J Bone Joint Surg 73:1418–1428
49. **Hauschka PV, Maurakos AE, Lafrati MD, Doleman SE, Klagsbrun M** 1986 Growth factors in bone matrix. J Biol Chem 261:12665–22674
50. **Robey PG, Young MF, Flanders KC, Roche NS, Kodaiah P, Reddi AH, Termine JD, Sporn MB, Roberts AB** 1987 Osteoblasts synthesize and respond to transforming growth factor-type b (TGF-B) *in vitro*. J Cell Biol 105:457–463
51. **Seitz PK, Zhu B-T, Cooper CW** 1992 Effect of transforming growth factor b on parathyroid hormone receptor binding and cAMP formation in rat osteosarcoma cells. J Bone Miner Res 7:541–546
52. **Centrella M, Massague J, Canalis E** 1986 Human Platelet-derived transforming growth factor-β stimulates parameters of bone growth in fetal rat calvariae. Endocrinology 119:2306–2312
53. **Dijke P, Iwata K, Goddard C, Pieler C, Canalis E, McCarthy TL, Centrella M** 1990 Recombinant transforming growth factor type beta 3: biological activities and receptor-binding properties in isolated bone cells. Mol Cell Biol 10:4473–4479
54. **Noda M, Camilliere J** 1989 *In vivo* stimulation of bone formation by transforming growth factor-β. Endocrinology 124:2991–2994
55. **Marcelli C, Yates AJ, Mundy GR** 1990 *In vivo* effects of human recombinant transforming growth factor beta on bone turnover in normal mice. J Bone Miner Res 5:1087–1096
56. **Mackie EJ, Trechsel U** 1990 Stimulation of bone formation *in vivo* by transforming growth factor-β: remodeling of woven bone and lack of inhibition by indomethacin. Bone 11:295–300
57. **Oreffo ROC, Mundy GR, Seyedin S, Bonewald LF** 1989 Activation of the bone derived latent TGFB complex by isolated osteoclasts. Biochem Biophys Res Commun 158:817–823
58. **Chenu C, Pfeilschifter J, Mundy GR, Roodman GD** 1988 Transforming growth factor beta inhibits formation of osteoclast-like cells in long term human marrow cultures. Proc Natl Acad Sci USA 85:5683–5687
59. **Mohan S, Baylink DJ** 1991 The role of insulin-like growth factor II in the coupling of bone formation to resorption. In: Spencer EM (ed) Modern Concepts of Insulin-Like Growth Factors. Elsevier Science Publishing Co., Loma Linda, CA, pp 169–184
60. **Zhang L, Leeman E, Carnes DC, Graves DT** 1991 Human osteoblasts synthesize and respond to platelet-derived growth factor. Am J Physiol 261:C348–C354
61. **Canalis E, Lorenzo J, Burgess WH, Maciag T** 1987 Effects of endothelial cell growth factor on bone remodelling *in vitro*. J Clin Invest 79:52–58
62. **Canalis E, McCarthy T, Centrella M** 1988 Effects of basic fibroblast growth factor on bone formation *in vitro*. J Clin Invest 81:1572–1577
63. **Tam CS, Heersche JNM, Murray TM, Parsons JA** 1982 Parathyroid hormone stimulates the bone apposition rate independently of its resorptive action: differential effects of intermittent and continuous administration. Endocrinology 110:506–512
64. **Hock JM, Hummert JR, Boyce R, Fonseca J, Raisz LG** 1989 Resorption is not essential for the stimulation of bone growth by hPTH-(1–34) in rats *in vivo*. J Bone Miner Res 4:449–458

64a. **Fujimori A, Cheng S-L, Avioli LV, Civitelli R** 1992 Structure-function relationship of parathyroid hormone: activation of phospholipase-C, protein kinase-A and -C in osteosarcoma cells. Endocrinology 130:29–36

65. **Rosenblatt M** 1986 Peptide hormone antagonists that are effective *in vivo*. N Engl J Med 315:1004–1013
66. **Rodan SB, Rodan GA** 1986 Dexamethasone effects on beta-adrenergic receptors and adenylate cyclase regulatory proteins G and G in ROS 17/2.8 cells. Endocrinology 118:2510–2518
67. **Yamaguchi DT, Hahn TJ, Iida-Klein A, Kleeman CR, Muallem S** 1987 Parathyroid hormone-activated calcium channels in an osteoblast-like clonal osteosarcoma cell line. cAMP-dependent and cAMP-independent calcium channels. J Biol Chem 262:7711–7718
68. **Cosman F, Morrow B, Kopal M, Bilezikian JP** 1989 Stimulation of inositol phosphate formation in ROS 17/2.8 cell membranes by guanine nucleotide, calcium and parathyroid hormone. J Bone Miner Res 4:413–420
69. **Fujimori A, Cheng S-L, Avioli LV, Civitelli R** 1991 Dissociation of second messenger activation by parathyroid hormone fragments in osteosarcoma cells. Endocrinology 128:3032–3039
70. **Klein RF, Nissenson RA, Strewler GJ** 1988 Forskolin mimics the effects of calcitonin but not parathyroid hormone on bone resorption *in vitro*. Bone Miner 4:247–256
71. **Murrills RJ, Dempster DW** 1990 The effects of stimulators of intracellular cyclic AMP on rat and chick osteoclasts *in vitro*: validation of a simplified light microscope assay of bone resorption. Bone 11:333–344
72. **Murrills RJ, Stein LS, Horbert WR, Dempster DW** 1992 Effects of phorbol myristate acetate on rat and chick osteoclasts. J Bone Miner Res 7:415–423
73. **Su Y, Chakraborty M, Nathanson MH, Baron R** 1992 Differential effects of the 3′,5′-cyclic adenosine monophosphate and protein kinase C pathways on the response of isolated rat osteoclasts to calcitonin. Endocrinology 131:1497–1502
74. **Teti A, Colucci S, Grano M, Argentino L, Zallone AZ** 1992 Protein kinase C affects microfilaments, bone resorption and $[Ca3^{+}]_0$ sensing in cultured osteoclasts. Am J Physiol 263:C130-C139
75. **Civitelli R, Hruska KA, Shen V, Avioli LV** 1990 Cyclic AMP-dependent and calcium dependent signals in parathyroid hormone function. Exp Gerontol 25:223–231

76. **Reid R, Lowe C, Cornish J, Gray DH, Skinner SJM** 1990 Adenylate cyclase blockers dissociate PTH-stimulated bone resorption from cAMP production. Am J Physiol 258:E708-E714
77. **Reid IR, Civitelli R, Avioli LV, Hruska KA** 1988 Parathyroid hormone depresses cytosolic pH and DNA synthesis in osteoblast-like cells. Am J Physiol 255:E9-E15
78. **Sugimoto T, Kano J, Fukase M, Fujita T** 1992 Second messenger signaling in the regualtion of cytosolic pHand DNA synthesis by parathyroid hormone (PTH) and PTH-related peptide in osteoblastic osteosarcoma cells: role of NA^+/H^+ exchange. J Cell Physiol 152:28–34
79. **Lowik CWGM, Van Leeuwen JPTM, van der Meer JM, Van Zeeland JK, Scheven BAA, Herrmann-Erlee MPM** 1985 A two-receptor model for the action of parathyroid hormone on osteoblast: a role for intracellular free calcium and cAMP. Cell Calcium 6:311–326
80. **Berthelson S, Pettinger WA** 1977 A functional basis for the classification of alpha-adrenergic receptors. Life Sci 21:595–606
81. **Fitzpatrick LA, Coleman DT, Bilezikian JP** 1992 The target tissue actions of parathyroid hormone. In: Coe FL, Favus MJ (eds) Disorders of Bone and Mineral Metabolism. Raven Press, New York, pp 123–148
82. **Garcia JC, McConkey CL, Martin KJ** 1989 Separate binding sites for intact PTH 1–84 and synthetic PTH 1–34 in canine kidney. Calcif Tissue Int 44:214–219
83. **Finkelman RD, Mohan S, Linkhart TA, Abraham SM, Boussy JP, Baylink DJ** 1992 PTH stimulates the proliferation of TE-85 human osteosarcoma cells by a mechanism not involving either increased cAMP or increased secretion of IGF-I, IGF-II or TGF B. Bone Miner 16:89–100
84. **Murrills RJ, Stein LS, Fey CP, Dempster DW** 1990 The effects of parathyroid hormone (PTH), PTH-related peptide on osteoclast resorption of bone slices *in vitro*: an analysis of pit size and the resorption focus. Endocrinology 127:2648–2653
85. **Fenton AJ, Kemp BE, Hammonds RG, Mitchelhill K, Moseley JM, Martin TJ, Nicholson GC** 1991 A potent inhibitor of osteoclastic bone resorption within a highly conserved pentapeptide region of parathyroid hormone-related protein, PTHrP (107–111). Endocrinology 129:3424–3426
86. **Pugsley LI, Selye H** 1933 The histological changes in the bone responsible for the action of parathyroid hormone on the calcium metabolism of the rat. J Physiol (Lond) 79:113–117
87. **Jaffe HL** 1933 Hyperparathyroidism (Recklinghausen's disease of bone). Arch Pathol 16:63–112
88. **Burrows RB** 1938 Variations produced in bones of growing rats by parathyroid extracts. Am J Anat 62:237–290
89. **Shelling DH, Asher DE, Jackson DA** 1933 Calcium, phosphorus studies: effects of variations and dosage of parahormone and of calcium and phosphorus and diet on concentrations of calcium and inorganic phosphorus in serum and on histology and chemical composition of bones of rats. Bull Johns Hopkins Hosp 53:348–389
90. **Heller M, Mclean FC, Bloom W** 1950 Cellular transformations in mammalian bones induced by parathyroid extract. Am J Anat 87:315–339
91. **Kalu DN, Pennock J, Doyle FH, Foster GV** 1970 Parathyroid hormone and experimental osteosclerosis. Lancet 27:1363–1366
92. **Walker DG** 1971 The induction of osteoporotic changes in hypophysectomized, thyroparathyroidectomized and intact rats of various ages. Endocrinology 89:1389–1406
93. **Schulz A, Remagen W, Grosse P** 1971 Effects of parathyroid extract on calcium metabolism and on bone morphology in the intact rat. Z Gesamte Exp Med 155:87–97
94. **Cramer CF, Suiker AP, Copp DH** 1961 Parathyroid influence on calcium and phosphorus adsorption by the gut. In: Greep RO, Talmage RV (eds) The Parathyroids. Charles C Thomas, Springfield, IL, pp 158–166
95. **Parsons JA** 1976 Parathyroid physiology and the skeleton. In: Bourne GH (ed) The Biochemistry and Physiology of Bone. Academic Press, London, pp 159–226
96. **Reeve J, Meunier PJ, Parsons JA, Bernat M, Bijvoet OLM, Courpron P, Edouard C, Klenerman L, Neer RM, Renier JC, Slovik D, Vismans EJFE, Potts JT** 1980 Anabolic effect of human PTH on trabecular bone in involutional osteoporosis: a multicenter trial. Br Med J 280:1340–1344
97. **Reeve J, Davies UM, Hesp R, McNally E, Katz D** 1990 Treatment of osteoporosis with human parathyroid peptide and observations on effect of sodium fluoride. Br Med J 301:314–318
98. **Hesp R, Hulme P, Williams D, Reeve J** 1981 The relationship between changes in femoral bone density and calcium balance in patients with involutional osteoporosis treated with human PTH fragment 1–34. Metab Bone Dis Rel Res 2:331–334
99. **Podbesek R, Edouard C, Meunier PJ, Parsons JA, Reeve J, Stevenson RW, Zanelli JM** 1983 Effects of two treatment regimes with synthetic human parathyroid hormone fragment on bone formation and the tissue balance of trabecular bone in greyhounds. Endocrinology 112:1000–1006
100. **Podesek R, Stevenson R, Zanelli GD, Edovard C, Meunier PJ, Reeve J, Parsons JA** 1980 Treatment with human parathyroid hormone fragment (hPTH 1–34) stimulates bone formation and intestinal calcium absorption in the greyhound: comparison with data from the osteoporosis trial. In: Hormonal Control of Calcium Metabolism. Excerpta Medica, Amsterdam, pp 118–123
101. **Hefti E, Trechsel U, Bonjour JP, Fleisch H, Schenk R** 1982 Increase of whole body calcium and skeletal mass in normal and osteoporotic adult rats treated with PTH. Clin Sci 62:389–396
102. **Gunness-Hey M, Hock JM** 1984 Increased trabecular bone mass in rats treated with synthetic human PTH. Metab Bone Dis Rel Res 5:177–181
103. **Hori M, Uzawa T, Morita K, Noda T, Takahashi H, Inoue J** 1988 Effect of human parathyroid hormone (PTH(1–34)) on experimental osteopenia of rats induced by ovariectomy. Bone Miner 3:193–199
104. **Hock JM, Gera I, Fonseca J, Raisz LG** 1988 Human PTH (1–34) increases bone in ovariectomized and orchidectomized rats. Endocrinology 122:2899–2904
105. **Hock JM, Hummert J, Boyce R, Fonseca J, Raisz LG** 1989 Resorption is not essential for the stimulation of bone growth by human PTH 1–34 in rats *in vivo*. J Bone Miner Res 4:449–458
106. **Gunness-Hey M, Gera I, Fonseca J, Raisz LG, Hock JM** 1988 1,25 Dihydroxyvitamin D3 alone or in combination with parathyroid hormone does not increase bone mass in young rats. Calcif Tissue Int 43:284–288
107. **Hock JM, Fonseca J** 1990 Anabolic effect of human synthetic PTH depends on growth hormone. Endocrinology 127:1804–1810
108. **Shen V, Dempster DW, Birchman R, Xu R, Lindsay R** 1993 Loss of cancellous bone mass and connectivity in ovariectiomized rats can be restored by combined treatment with parathyroid hormone and estradiol. J Clin Invest, 91:2479–2487
109. **Tada K, Yamamuro T, Okumura H, Kasai R, Takahashi H** 1990 Restoration of axial and appendicular bone volumes by h-PTH (1-34) in parathyroidectomized and osteopenic rats. Bone 11:163–169
110. **Mellish RWE, Shen V, Birchman R, Meng XW, Horbert W, Dempster D, Lindsay R** 1990 Quantitative analysis of trabecular bone in ovariectomized rats after intermittent administration of low dose human parathyroid hormone fragment (1–34) and 17β-estradiol. In: Christiansen C, Overgaard K (eds) Osteoporosis 1990. Handestrykkeriet Aalborg APS, Aalborg, Denmark, pp 1335–1337
111. **Gunness-Hey M, Hock JM** 1989 Loss of the anabolic effect of PTH on bone after discontinuation of hormone in rats. Bone 10:447–452
112. **Liu CC, Kalu DN** 1990 Human parathyroid hormone (1–34) prevents bone loss, augments bone formation in sexually mature ovariectomized rats. J Bone Miner Res 5:973–982
113. **Liu CC, Kalu DN, Salerno E, Echon R, Hollis BW, Ray M** 1991 Preexisting bone loss associated with ovariectomy in rats is reversed by PTH. J Bone Miner Res 6:1071–1080
114. **Mosekilde L, Sogaard CH, Danielson CC, Torring O, Nilsson MHL** 1991 The anabolic effects of human PTH on rat vertebral body mass are also reflected in the quality of bone, assessed by biomechanical testing: a comparison study between hPTH (1–34) and hPTH (1–84). Endocrinology 129:421–428
115. **Wronski TJ, Cintron M, Dann LM** 1988 Temporal relationship between bone loss and increased bone turnover in ovariectomized rats. Calcif Tissue Int 43:179–183
116. **Ibbotson KJ, Orcutt CM, D'Souza SM, Paddock CL, Arthur JA, Jankowsky ML, Boyce RW** 1992 Contrasting effects of PTH and

IGF-1 in an aged OVX rat model of postmenopausal osteoporosis. J Bone Miner Res 7:425–432
117. **Tuukkanen J, Jalovaara P, Vaananen K** 1990 Calcitonin treatment of immobilization osteoporosis rats. Acta Physiol Scand 141:119–124
118. **Kalu DN, Echon R, Hollis BW** 1990 Modulation of ovariectomy-related bone loss by parathyroid hormone in rats. Mech Ageing Dev 56:49–62
119. **Ejeersted C, Andreassen TT, Oxlund H, Jorgensen PH, Bak B, Nilsson M, Torring O,** PTH (1–34) and (1–84) increase the mechanical strength and thickness of cortical bone. Program of the Second Rat Skeleton Workshop, Chicago, IL, 1992 (Abstract)
120. **Kalu DN** 1991 The ovariectomized rat model of postmenopausal bone loss. Bone Miner 15:175–191
121. **Frost HM, Jee WSS** 1992 On the rat model of human osteopenias and osteoporosis. Bone Miner 18:227–236
122. **Malluche HH, Sherman D, Meyer W, Ritz E, Norman AW, Massry SG** 1982 Effects of long term infusion of physiologic doses of 1–34 PTH on bone. Am J Physiol 242:F197–F202
123. **Hock JM, Gera I** 1992 Effects of continuous and intermittent administration and inhibition of resorption on the anabolic response of bone to PTH. J Bone Miner Res 7:65–72
124. **Cheng PT, Bader SM** 1990 Effects of fluoride on rat cancellous bone. Bone Miner 11:153–161
125. **Mori S, Jee WSS, Li XJ, Chan S, Kimmel DB** 1990 Effects of prostaglandin E_2 on production of new cancellous bone in the axial skeleton of ovariectomized rats. Bone 11:103–113
126. **Ke HZ, Li XJ, Jee WSS** 1991 Partial loss of anabolic effect of prostaglandin E_2 on bone after its withdrawal in rats. Bone 12:173–183
127. **Erben RG, Weiser H, Sinowatz F, Rambeck WA, Zucker H** 1992 Vitamin D metabolites prevent vertebral osteopenia in ovariectomized rats. Calcif Tissue Int 50:228–236
128. **Jerome CP,** Comparison of effects of growth hormone (GH), growth hormone releasing factor peptide (GRF), transforming growth factor beta 2 (TGF beta), PTH and PTH+GH on bone formation and bone mass in retired breeder female rats. Program of the Second Rat Skeleton Workshop, Chicago, IL, 1992 (Abstract)
129. **Muller K, Cortesi R, Jeker H,** Effects of PTH, IGF-1 and TGF beta 2 on trabecular and cortical bone in the intact and ovx rats. Program of the Second Rat Skeleton Workshop, Chicago, IL, 1992 (Abstract)
130. **Theun HM, Roholl PJM, Spencer EM,** PTH, IGF-1 effects in an aging rat model for postmenopausal osteoporosis. Program of the Second Rat Skeleton Workshop, Chicago, IL, 1992 (Abstract)
131. **Clemmons DR** 1992 Editorial: role of insulin-like growth factor-1 in reversing catabolism. J Clin Endocrinol Metab 75:1183–1185
132. **McGuire JL, Marks SCJ** 1974 The effects of PTH on bone cell structure and function. Clin Orthop 100:392–405
133. **Gera I, Hock JM, Gunness-Hey M, Fonseca J, Raisz LG** 1987 Indomethacin does not inhibit the anabolic effect of PTH on the long bones of rats. Calcif Tissue Int 40:206–211
134. **Pocock NA, Eisman JA, Gwinn T, Sambrook P, Kelly P, Freund M, Yeates M** 1989 Muscle strength, physical fitness but not age predict femoral neck bone mass. J Bone Miner Res 4:441–448
135. **Norman AW** 1985 The vitamin D endocrine system. Physiologist 28:219–232
136. **Lawson DEM, Muir E** 1991 Molecular biology and vitamin D function. Proc Nutr Soc 50:131–137
137. **Lindgren U, DeLuca HF** 1982 Role of parathyroid hormone and 1,25-dihydroxyvitamin D_3 in the development of osteopenia in oophorectomized rats. Calcif Tissue Int 34:510–514
138. **Tam CS, Jones G, Heersche JNM** 1981 The effect of vitamin D restriction and repletion on bone apposition in the rat and its dependence on PTH. Endocrinology 109:1448–1453
138a. **Dempster DW, Lindsay R** 1993 Pathogenesis of osteoporosis. Lancet 341:797–801
138b. 1993 Consensus Development Conference Statement from the Fourth International Symposium on Osteoporosis. Am J Med, in press
139. **von Recklinghausen F** 1891 Die fibrose oder deformirende Ostitis die Osteomalacie und die carcinose in gengensitigen. In: Greiner G (ed) Beziehungen. Festschrift Rudolf Virchow, Berlin
140. **Mandl R** 1925 Therapeutisher verusch bei ostitis fibrosa generalizata mittels extirpation eines epithelkorperchen tumors. Wien Klin Wochenschr 38:1383
141. **Albright F, Aub JC, Bauer W** 1934 Hyperparathyroidism: a common and polymorphic condition as illustrated by seventeen proven cases from one clinic. JAMA 102:1276
142. **Albright F, Reifenstein EC** 1948 The Parathyroid Glands and Metabolic Bone Disease. Williams & Wilkins, Baltimore, MD
143. **Murray TM** 1989 Parathyroid hormone and hyperparathyroidism. In: Heersche JNM (ed) Metabolic Bone Disease: Cellular and Tissue Mechanisms. CRC Press, Boca Raton, FL, pp 105–133
144. **Uehlinger E** 1940 Osteofibrosis deformans juvenilis. Virchows Arch [A] 306:255
145. **Jaffe HL** 1968 Tumors and Tumorous Conditions of the Bone and Joints. Lea & Febiger, Malvern, PA
146. **Schajowicz F** 1981 Tumors and Tumor-like Lesions of Bones and Joints. Springer Verlag, Berlin
147. **Parfitt AM** 1976 The actions of parathyroid hormone on bone. Metabolism 25:1033–1069
148. **Parfitt AM, Oliver I, Villanueva ADR** 1979 Bone histology in metabolic bone disease. Orthop Clin North Am 10:329–345
149. **Mallette LE, Bilezikian JP, Heath DA, Aurbach GD** 1974 Primary hyperparathyroidism: clinical and biochemical features. Medicine 53:127–146
150. **Keating RF** 1961 Diagnosis of primary hyperparathyroidism. JAMA 178:547–555
151. **Boonstra CE, Jackson CE** 1965 Hyperparathyroidism detected by routine serum calcium analysis. Ann Intern Med 63:468–474
152. **Purnell DC, Smith LH, Scholz DA** 1971 Primary hyperparathyroidism: a prospective clinical study. Am J Med 50:670–678
153. **Lafferty FW** 1981 Primary hyperparathyroidism: changing clinical spectrum, prevalence of hypertension and discriminant analysis of laboratory tests. Arch Intern Med 141:1761–1766
154. **Mundy GR, Cove DH, Fisken R** 1980 Primary hyperparathyroidism: changes in the pattern of clinical presentation. Lancet 1:1318–1320
155. **Heath H, Hodgson SF, Kennedy MA** 1980 Primary hyperparathyroidism incidence, morbidity, and potential economic impact in a community. N Engl J Med 302:189–193
156. **Rao SD** 1985 Primary hyperparathyroidism: changing patterns in presentation and treatment decisions in the eighties. Henry Ford Hosp Med J 33:194–197
157. **Dempster DW, Feldman F, Seldin D, Jacobs TP, Sirise Cafferty M, Parisien MV, Lindsay R, Clemens TL, Bilezikian JP** 1989 Skeletal disease in primary hyperparathyroidism. J Bone Miner Res 4:283–291
158. **Dauphine RT, Riggs BL, Scholz DA** 1975 Back pain and vertebral crush fractures: an unemphasized mode of presentation for primary hyperparathyroidism. Ann Intern Med 83:365–367
159. **Kochersberger G, Buckley NJ, Leight GS, Martinez S, Studenski S, Vogler J, Lyles KW** 1987 What is the clinical significance of bone loss in primary hyperparathyroidism. Arch Intern Med 147:1951–1953
160. **Peacock M, Horsman A, Aaron JE, Marshall DH, Selby PL, Simpson M** 1984 The role of parathyroid hormone in bone loss. In: Christiansen C, Arnaud CD, Nordin BEC, Parfitt AM, Peck WA, Riggs BL (eds): Osteoporosis. Proceedings of the 1984 Copenhagen International Symposium on Osteoporosis. Aalborg Stiftsbogtrykkeri, Copenhagen, pp 463–467
161. **Martin P, Bergmann P, Gillet C, Fuss M, Kinnaert P, Corvilain J, van Geertrugden J** 1986 Partially reversible osteopenia after surgery for primary hyperparathyroidism. Arch Intern Med 146:689–691
162. **Richardson ML, Pozzi-Mucelli RS, Kanter AS, Kolb FO, Ettinger B, Genant HK** 1986 Bone mineral changes in primary hyperparathyroidism. Skeletal Radiol 15:85–95
163. **Kleerekoper M, Villaneuva AR, Mathews CHE** 1983 PTH mediated bone loss in primary, secondary hyperparathyroidism. In: Frame B, Potts JT (eds) Clinical Disorders of Bone and Mineral Metabolism. Excerpta Medica, Amsterdam, pp 200–203
164. **Rao DS, Wilson RJ, Kleerekoper M, Parfitt AM** 1988 Lack of biochemical progression or continuation of accelerated bone loss in mild asymptomatic primary hyperparathyroidism: evidence for biphasic disease course. J Clin Endocrinol Metab 67:1294–1298
165. **Parfitt AM** 1986 Accelerated cortical bone loss: primary and

secondary hyperparathyroidism. In: Uhthoff H, Stahl E (eds) Current Concepts of Bone Fragility. Springer Verlag, Berlin, pp 279–285

166. **Parfitt AM** 1989 Surface specific bone remodeling in health and disease. In: Kleerekoper M (ed) Clinical Disorders of Bone and Mineral Metabolism. Mary Ann Liebert, Inc., New York, pp 7–14
167. **Parisien M, Silverberg SJ, Shane E, de la Cruz L, Lindsay R, Bilezikian JP, Dempster DW** 1990 The histomorphometry of bone in primary hyperparathyroidism: preservation of cancellous bone structure. J Clin Endocrinol Metab 70:930–938
168. **Seeman E, Wahner HW, Offord KP, Kumar R, Johnson WJ, Riggs BL** 1982 Differential effects of endocrine dysfunction on the axial and the appendicular skeleton. J Clin Invest 69:1302–1309
169. **Wilson RJ, Rao DS, Ellis B, Kleerekoper M, Parfitt AM** 1988 Mild asymptomatic primary hyperparathyroidism is not a risk factor for vertebral fractures. Ann Intern Med 109:959–962
170. **Lafferty FW, Hubay CA** 1989 Primary hyperparathyroidism: a review of the long term surgical and non-surgical morbidities as a basis for a ration approach to treatment. Arch Intern Med 149:789–796
171. **Melton III LJ, Atkinson EJ, O'Fallon WM, Heath HI** 1992 Risk of age-related fractures in patients with primary hyperparathyroidism. Arch Intern Med 152:2269–2273
172. **Mosekilde L, Melsen F** 1978 A tetracycline-base histomorphometric evaluation of bone resorption and bone turnover in hyperthyroidism and hyperparathyroidism. Acta Med Scand 204:97–102
173. **Melsen F, Mosekilde L** 1981 The role of bone biopsy in the diagnosis of metabolic bone disease. Orthop Clin North Am 12:571–602
174. **Eriksen EF** 1986 Normal, pathological remodeling of human trabecular bone: three dimensional reconstruction of the remodeling sequence in normals and in metabolic bone disease. Endocr Rev 7:379–408
175. **Delmas PD, Meunier PJ, Faysse E, Saubier EC** 1986 Bone histomorphometry and serum bone gla-protein in the diagnosis of primary hyperparathyroidism. World J Surg 10:572–578
176. **Delling G** 1987 Bone morphology in primary hyperparathyroidism. A qualitative and quantitative study of 391 cases. Appl Pathol 55:147–159
177. **de Vernejoul MC, Benamount MP, Cancela L** 1988 Hyperparathyroidie primitive vue en rhumatologie: signes cliniques et relations entre les signes histologiques osseux et les parametres biologiques. Rev Rhum 55:489–494
178. **Baron R, Magee SS** 1983 Estimation of trabecular bone resorption by histomorphometry: evidence for a prolonged reversal phase with normal resorption in post menopausal osteoporosis and coupled increased resorption in primary hyperparathyroidism. In: Frame B, Potts JT (eds) Clinical Disorders of Bone and Mineral Metabolism. Excerpta Medica, Amsterdam, pp 191–195
179. **Riggs BL, Kelly PJ, Jowsey J, Keating FR** 1965 Skeletal alterations in hyperparathyroidism: determination of bone formation, resorption, and morphologic changes by microradiography. J Clin Endocrinol Metab 25:777–783
180. **Byers PD, Smith R** 1971 Quantitative histology of bone in hyperparathyroidism: its relation to clinical features, x-ray, and biochemistry. Q J Med 160:471–486
181. **Wilde CD, Jaworski ZF, Villaneuva AR, Frost HM** 1973 Quantitative histological measurements of bone turnover in primary hyperparathyroidism. Calcium Tissue Res 12:137–142
182. **Meunier PJ, Vignon G, Bernard J** 1973 Clinical aspects of metabolic bone disease. In: Frame B, Parfitt AM, Duncan H (eds) Quantitative Bone Histology as Applied to the Diagnosis of Hyperparathyroid States. Excerpta Medica, Amsterdam, pp 215–221
183. **Eriksen EF, Mosekilde L, Melsen F** 1973 Trabecular bone remodeling and balance in primary hyperparathyroidism. Bone 7:213–221
184. **Christiansen P, Steiniche T, Vesterby A, Mosekilde L, Hessov I, Melsen F** 1992 Primary hyperparathyroidism: iliac crest trabecular bone volume, structure, remodeling, and balance evaluated by histomorphometric methods. Bone 13:41–49
185. **Doyle FH** 1966 Some quantitative radiological observations in primary and secondary hyperparathyroidism. Br J Radiol 39:161–167
186. **Ellis K, Hostim RJ** 1960 The skull in hyperparathyroid bone disease. Am J Roentgenol Radium Ther Nucl Med 83:732–740
187. **Jaffe HL** 1933 Hyperparathyroidism (Recklinghausen's disease of bone). Arch Pathol 16:63–112
188. **Aitken RE, Kerr JL, Lloyd HM** 1964 Primary hyperparathyroidism with osteosclerosis and calcification in articular cartilage. Am J Med 37:813–820
189. **Eugenidis N, Olah AJ, Haas HG** 1972 Osteosclerosis in hyperparathyroidism. Radiology 105:265–275
190. **Genant HK, Baron JM, Straus FH, Paloyan E, Jowsey J** 1975 Osteosclerosis in primary hyperparathyroidism. Am J Med 59:104–113
191. **van Holsbeeck M, Roex L, Favril A, Burssens D, Baert AL** 1987 Osteosclerosis in primary hyperparathyroidism. Fortschr Rontgenstr 147:690–691
192. **Dresser R** 1933 Osteitis fibrosa cystica associated with parathyroid overactivity. Am J Roentgenol Radium Ther Nucl Med 30:596–603
193. **Albright F, Baird PC, Cope D, Bloomberg E** 1934 Studies on the physiology of the parathyroid glands. IV. Renal complication of hyperparathyroidism. Am J Med Sci 187:49–65
194. **Aub JC,** Lack of effect of parathyroid gland on growth of bone: case presentation. In: Proceedings of the 26th Annual Meeting of the Endocrine Society, Atlantic City, NJ, 1942, p 1024
195. **Shallow TA, Fry KE** 1948 Parathyroid adenoma. Occurrence in father and daughter. Surgery 24:1020–1025
196. **Adam A, Ritchie D** 1954 Hyperparathyroidism with increased bone density in the areas of growth. J Bone Joint Surg 36B:257–260
197. **Lloyd HM, Aitken RE, Ferrier TM** 1965 Primary hyperparathyroidism resembling rickets of late onset. Br Med J 2:853–856

197a. **Parisien M, Mellish RWE, Silverberg SJ, Shane E, Lindsay R, Bilezikian JP, Dempster DW** 1992 Maintenance of cancellous bone connectivity in primary hyperparathyroidism: trabecular strut analysis. J Bone Miner Res 7:913–919

198. **Wakamatsu E, Sissons HA** 1969 The cancellous bone of the iliac crest. Calcif Tissue Res 4:147–161
199. **Parfitt AM, Mathews HE, Villaneuva AR, Kleerekoper M, Frame B** 1983 Relationships between surface, volume, and thickness of iliac trabecular bone in aging and in osteoporosis. Implications for the microanatomic and cellular mechanisms of bone loss. J Clin Invest 72:1396–1409
200. **Garrahan N, Mellish R, Compston J** 1986 A new method for the two-dimensional analysis of bone structure in human iliac crest biopsies. J Microsc 142:341–349
201. **Mellish RWE, Ferguson-Pell MW, Cochran GVB, Lindsay R, Dempster DW** 1991 A new method for the two-dimensional analysis of bone structure in human iliac crest biopsies. J Bone Miner Res 6:689–696
202. **Vesterby A** 1990 Star volume of marrow space and trabeculae in iliac crest. Sampling procedure and correlation to star volume of first lumbar vertebra. Bone 11:149–155
203. **Parfitt AM** 1987 Trabecular bone architecture in the pathogenesis and prevention of fracture. Am J Med 82:68–72
204. **Parisien MV, McMahon D, Pushparaj N, Dempster DW** 1988 Trabecular architecture in iliac crest bone biopsies: intra-individual variability in structural parameters and changes with age. Bone 9:289–295
205. **Kleerekoper M, Villanueva AR, Stanciu J, Rao DS, Parfitt AM** 1985 The role of three-dimensional trabecular microstructure in the pathogenesis of vertebral compression fractures. Calcif Tissue Int 37:594–597
206. **Aaron JE, Makins NB, Sagreiya K** 1987 The microanatomy of trabecular bone loss in normal aging men and women. Clin Orthop 215:260–271
207. **Parisien M, Recker R, Silverberg SJ, Shane E, Mellish RWE, Lindsay R, Bilezikian JP, Dempster DW** 1990 Cancellous bone structure in postmenopausal women with primary hyperparathyroidism. In: Christiansen C, Overgaard K (eds) Third International Symposium on Osteoporosis. Osteopress ApS, Copenhagen, pp 1139–1140
208. **Parisien M, Schnitzer M, Nieves J, Mellish RW, Silverberg SJ, Shane E, Recker R, Kimmel D, Cosman F, Bilezikian JP, Lindsay R, Dempster DW,** A comparison of bone structure and turnover in postmenopausal women with osteoporosis or primary hyper-

parathyroidism. In: Proceedings of the Fourth International Symposium on Osteoporosis, Hong Kong, 1993, pp 162–163
209. **Courpron P, Meunier P, Bressot C, Giroux JM** 1976 Amount of bone in iliac crest biopsy: significance of the trabecular bone volume. Its values in normal and in pathological conditions. In: Meunier PJ (ed) Proceedings of Bone Histomorphometry. Lyon Second International Workshop, Lyon, France, pp 39–53
210. **Charhon SA, Edouard CM, Arlot ME, Meunier PJ** 1982 Effects of parathyroid hormone on remodeling of iliac trabecular bone packets in patients with primary hyperparathyroidism. Clin Orthop 162:255–263
211. **Hesp R, Reeve J** 1990 Letter to the editor. J Bone Miner Res 5:103
212. **Parisien M, Dempster DW, Silverberg SJ, Shane E, Seldin D, Lindsay R, Bilezikian JP** 1990 Relationship between bone mass as assessed by densitometry and by histomorphometry in primary hyperparathyroidism. J Bone Miner Res 5:[Suppl 2]:S108
213. **Parisien M, Cosman F, Silverberg SJ, Shane E, Lindsay R, Bilezikian JP, Dempster DW** 1992 Relationship between bone mass by densitometry and by histomorphometry in primary hyperparathyroidism and in osteoporosis. Bone Miner 17[Suppl 1]:214(Abstract)
214. **Reeve J, Hesp R, Williams D, Hulme P, Klenerman L, Zanelli JM, Darby AJ, Tregear GW, Parsons JA** 1976 Anabolic effect of low doses of a fragment of human parathyroid hormone on the skeleton in postmenopausal osteoporosis. Lancet 1:1035–1038
215. **Reeve J, Tregear GW, Parsons JA** 1976 Preliminary trial of low doses of human parathyroid hormone 1–34 peptide in treatment of osteoporosis. Calcif Tissue 21:469–477
216. **Niall HD, Sauer RT, Jacobs JW, Keutmann HT, Segre GV, O'Riordan JLH, Aurbach GD, Potts Jr JT** 1974 The amino acid sequence of the amino terminal 37 residues of human parathyroid hormone. Proc Natl Acad Sci USA 71:384–388
217. **Tregear GW, VanRietschoten J, Greene E, Keutmann HT, Niall HD, Reit B, Parsons JA, Potts Jr JT** 1974 Bovine parathyroid hormone: minimum chain length of synthetic peptide required for biological activity. Endocrinology 93:1349–1353
218. **Melick RA, Gill Jr JR, Berson SA, Yalow RS, Bartter FC, Potts Jr JT, Aurbach GD** 1967 Antibodies and clinical resistance to parathyroid hormone. N Engl J Med 276:144–147
219. **Slovik DM, Neer RM, Potts Jr JT** 1981 Short-term effects of synthetic human parathyroid hormone (1–34) administration on bone mineral metabolism in osteoporotic patients. J Clin Invest 68:1261–1271
220. **Reeve J, Arlot M, Bernat M, Charhon S, Edouard C, Slovik D, Vismans FJFE, Meunier PJ** 1981 Calcium-47 kinetic measurements of bone turnover compared to bone histomorphometry in osteoporosis: the influence of human parathyroid fragment (hPTH 1–34) therapy. Metab Bone Dis Rel Res 3:23–30
221. **Reeve J, Arlot M, Bernat M, Edouard C, Hesp R, Slovik D, Vismans FJFE, Meunier PJ** 1980 Treatment of osteoporosis with human parathyroid hormone fragment 1–34: a positive final tissue balance in trabecular bone. Metab Bone Dis Rel Res [Suppl] 2:355
222. **Bilezikian JP, Canfield RE, Jacobs TP, Polay JS, D'Amano P, Eisman JA, DeLuca HF** 1978 Response of 1,25-dihydroxyvitamin D_3 to hypocalcemia in human subjects. N Engl J Med 299:437–441
223. **Kent GN, Loveridge N, Reeve J, Zanelli JM** 1985 Pharmacokinetics of synthetic human parathyroid hormone 1–34 in man measured by cytochemical bioassay and radioimmunoassay. Clin Sci 68:171–177
224. **Parsons JA, Meunier PJ, Neer RM, Podbesek R, Reeve J** 1981 Effects of synthetic human parathyroid hormone fragment (hPTH 1–34) on bone mass and bone mineral metabolism. In: DeLuca HF, Frost HM, Jee JSS, Johnston Jr CC, Parfitt AM (eds) Osteoporosis. University Park Press, Baltimore, MD, pp 457–465
225. **Slovik DM, Rosenthal DI, Doppelt SH, Potts Jr JT, Daly MA, Campbell JA, Neer RM** 1986 Restoration of spinal bone in osteoporotic men by treatment with human parathyroid hormone (1–34) and 1,25-dihydroxyvitamin D. J Bone Miner Res 1:377–381
226. **Neer RM, Slovik D, Doppelt S** 1987 The use of parathyroid hormone plus 1,25-dihydroxyvitamin D to increase trabecular bone in osteoporotic men and postmenopausal women. In: Christiansen C, Johansen JS, Riis BJ (eds): Osteoporosis 1987. Osteopress, Copenhagen, pp 829–835
227. **Neer M, Slovik DM, Daly N, Potts Jr JT, Nussbaum SR** 1993 Treatment of postmenopausal osteoporosis with daily parathyroid hormone plus calcitriol. Osteopor Int [Suppl] 1:S204–205
228. **Lindsay R, Cosman F, Nieves J, Dempster DW, Shen VA** 1993 Controlled trial of the effects of 1–34hPTh in estrogen treated osteoporotic women. J Bone Miner Res (Abstract), 8[Suppl. 1]:S130
229. **Hesch R-D, Busch U, Prokop M, Delling G, Rittinghaus E-F** 1989 Increase of vertebral density by combination therapy with pulsatile 1–38hPTH and sequential addition of calcitonin nasal spray in osteoporotic patients. Calcif Tissue Int 44:176–180
230. **Hesch RD, Heck J, Auf'mkolk B** 1984 First clinical observations with hPTH (1–38), a more potent human parathyroid hormone peptide. Horm Metab Res 169:559–560
231. **Reeve J, Bradbeer JN, Arlot M, Davies UM, Green JR, Hampton L, Edouard C, Hesp R, Hulme P, Ashby JP, Zanelli JM, Meunier PJ** 1991 hPTH 1–34 treatment of osteoporosis with added hormone replacement therapy: biochemical, kinetic and histological responses. Osteopor Int 1:162–170
232. **Bradbeer JN, Arlot ME, Meunier PJ, Reeve J** 1992 Treatment of osteoporosis with parathyroid peptide (hPTH 1–34) and oestrogen: increase in volumetric density of iliac cancellous bone may depend on reduced trabecular spacing as well as increased thickness of packets of newly formed bone. Clin Endocrinol (Oxf) 37:282–289
233. **Reeve J, Arlot ME, Bradbeer JN, Hesp R, McNally E, Meunier PJ, Zanelli JM** 1993 Human parathyroid peptide treatment of vertebral osteoporosis. Osteopor Int 3[Suppl]:199–203
234. **Hodsman AB, Fraher LJ, Ostbye T, Adachie JD, Steer BM** 1993 An evaluation of several biochemical markers for bone formation and resorption in a protocol utilizing cyclical parathyroid hormone and calcitonin therapy for osteoporosis. J Clin Invest, 91:1138–1148
235. **Reeve J, Arlot M, Price TR, Edouard C, Hesp R, Hulme P, Ashby JP, Zanelli JM, Green JR, Tellez M, Katz D, Spinks J, Meunier PJ** 1987 Periodic courses of human 1–34 parathyroid peptide alternating with calcitriol paradoxically reduce bone remodeling in spinal osteoporosis. Eur J Clin Invest 17:421–428
236. **Reeve J, Podbesek RD, Price TR, Arlot M, Bartlett C, Deacon AC, Edovard C, Green JR, Hesp R, Hulme P, Katz D, Tellez M, Zanelli GD, Zanelli JM, Meunier PJ** 1984 Studies of a "short-cycle" ADFR regime using parathyroid peptide hPTH 1–34 in idiopathic osteoporosis and in a dog model. In: Christiansen C, Arnaud CD, Nordin BEC, *et al.* (eds): Osteoporosis 1984. Aalborg Stiftsbogtrykkeri, Copenhagen, pp 567–573
237. **Hesch RD, Heck J, Delling G, Keck E, Reeve J, Canzler H, Schober O, Harms H, Rittinghaus EF** 1988 Results of a stimulatory therapy of low bone metabolism in osteoporosis with (1–38)hPTH and disphosphonate EHDP. Klin Wochenschr 66:976–984
238. **Hodsman AB, Fraher LJ** 1990 Biochemical responses to sequential human parathyroid hormone (1–38) and calcitonin in osteoporotic subjects. Bone Miner 9:137–152
239. **Hodsman AB, Steer BM, Fraher LJ, Drost DJ** 1991 Bone densitometric and histomorphometric responses to sequential human parathyroid hormone (1–38) and salmon calcitonin in osteoporotic patients. Bone Miner 14:67–83

Vol. 56, No. 3, 1974 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

FIBRIN GEL STRUCTURE: INFLUENCE OF CALCIUM AND COVALENT CROSS-LINKING ON THE ELASTICITY

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SUMMARY: Calcium ion causes the development of a higher elastic modulus in fibrinogen solutions clotted by thrombin. By also measuring the development of covalent crosslinks introduced by activated Factor XIII, we find that (a) calcium ion causes an overall increase of the modulus, (b) covalent crosslinking of α-chains causes an increase of the modulus, while (c) covalent crosslinking of γ-chains does not.

Introduction: The use of elasticity measurements as a tool in studying the clotting of fibrinogen solutions was initiated by Ferry and coworkers (1,2). The theoretical basis for this work is that the elastic modulus is a measure of 1) the stiffness of the strands making up the network of molecules in the gel and 2) the concentration of branchpoints of the network*. Both quantities are of interest, in that they tell us about the structure of the gel; and, furthermore, the elasticity can be measured readily in gels which are too opaque for the optical methods commonly used to study protein solutions. In order to make the elastic measurement a useful tool in a study of the clotting of fibrinogen, we have used a basically simple, but sensitive Couette elastometer, and have measured the elastic modulus of fibrin gels and of fibrinogen solutions clotting under the action of thrombin. We report here the difference in the elastic modulus when the fibrin molecules are covalently crosslinked, and when they are not. These crosslinks are introduced by an enzyme, activated plasma Factor XIII (3-5), which catalyzes the formation of

*Network branchpoints are commonly called crosslinks by polymer physicists. However, the word crosslinks is used by biochemists, and here by us, to describe the linkage of two protein molecules.

isopeptide bonds between ε-amino groups of lysine and γ-carboxyamide groups of glutamine and requires calcium ion for activity. Our fibrinogen preparation contains both Factor XIII and calcium. Thus, steps must be taken to remove all the free and tightly bound calcium. Then, by adding $CaCl_2$ to the solution, one can control the formation of covalent crosslinks in the clot.

Materials & Methods: Bovine fibrinogen was obtained from Miles laboratory. Most experiments reported here were done with lot 15, which we found to contain approximately three calcium ions per fibrinogen molecule. Earlier experiments were done with lot 14, which is calcium free by our assay. Both preparations were contaminated by Factor XIII as evidenced by the formation of covalent crosslinks upon clotting with purified thrombin (Parke-Davis). The tightly bound calcium was removed by dialysis of the fibrinogen stock solution (approximately 15 mg/ml) with 0.02 M EDTA at pH 8.0 for 10 hours at 4°C. The calcium content was assayed using a Model 306 Perkin-Elmer flame spectrophotometer. The elastic moduli were measured with a Couette elastometer (6) which had been slightly modified to permit measurements of lower moduli. Polyacrylamide gel electrophoresis in phosphate buffer pH 7.0, containing sodium dodecyl sulfate and dithiothreitol was performed according to described procedures (7).

Results & Discussion: It has been reported that calcium increases the rigidity of fibrin clots (1,2,8-10), and therefore effects due specifically to the covalent crosslinks and to differences in calcium concentration must be separated. Dialysis affects the structure of the fibrin clot. We find that the removal of tightly bound calcium ions irreversibly reduces the elasticity, in the presence of added calcium, to about 35% of the original value. The elasticity of the fibrin gel clotted from EDTA-treated fibrinogen (lot 15) is the same as that of a calcium-free fibrinogen preparation from the same source (lot 14). The existence of tightly bound calcium in fibrinogen has been demonstrated by many investigators. Endres and Scheraga (11) indicated that the fibrinogen molecule may possess multiple calcium binding sites, differing

in affinity. The work of these authors, as well as that of Godal (12,13), of Bithell (14) and of Blombäck et al. (15) indicates that the tightly bound calcium ions are essential constituents of fibrinogen and fibrin. Reversible changes occur in the fibrinogen molecule in the presence of EDTA, presumably because of the removal of calcium, which lead to an increase of the clotting time and a change in the sedimentation constant. The elasticity data show that there is an irreversible change, besides the reversible change noted by these authors, which persists when EDTA is removed and calcium is added back. All experiments described below regard the behavior of EDTA-treated fibrinogen, but in the absence of EDTA.

Fig. 1 shows the results of two parallel experiments, in which fibrinogen solutions were clotted in the elastometer in the presence and absence of calcium. At short times, the difference between the two curves increases approximately as the modulus itself. At longer times, the modulus of the

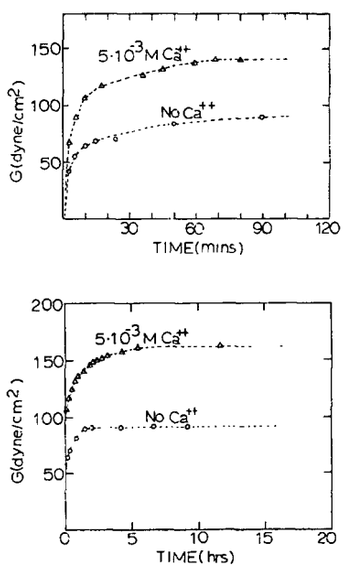

Fig. 1. Elastic modulus of fibrinogen solutions (1.5 mg/ml) clotted by the action of thrombin (1.25 units/ml), in the presence of $CaCl_2$ and without calcium ion, as a function of time (plotted using two different scales). Solvent: 0.1 M NaCl, 0.05 Tris buffer pH 7.4.

gel containing calcium continues to increase slowly when there no longer is any formation of fibrin from fibrinogen, as evidenced by the constancy of the modulus in the absence of calcium. The simplest explanation of these observations is that 1) the strands of fibrin molecules are stiffer when formed in the presence of calcium ion or of active Factor XIII and 2) the strands are further stiffened by chemical crosslinking after the network is complete. One could, for either or both explanations, suppose the presence of a larger concentration of branchpoints in the network for the same concentration of fibrin.

These conclusions are further clarified by polyacrylamide electrophoretic analysis of the covalent crosslinking of fibrin as a function of time, which allows the determination of relative concentrations of α-, β- and γ-chains of which the fibrin molecule is composed. In fibrin not containing added calcium, the concentration of α- and γ-chains varies with time; the γ-band disappears because of the formation of γ-dimer and the α-band disappears because α-polymers are formed. It has been shown that the β-chains of fibrin are not involved in crosslinking (16-18). In Fig. 2 we show photometer tracings of a series of runs as a function of time. Quantitative analysis of these data and another series gave the fraction of α- and γ-chain remaining as a function of time, reported in Fig. 2B and C.

In Fig. 2B and C, we also show the excess modulus due to the presence of calcium and the action of Factor XIII as obtained from the data of Fig. 1, plotted to such a scale as to obtain the best correspondence with analytical data. The excess rigidity observed at short times, which parallels the increase of the rigidity as clotting progresses, is not correlated with the progress of the chemical crosslinking reactions; neither is the disappearance of γ-chains correlated with differences in rigidity. However, the α-polymerization reaction progresses as does the excess rigidity observed at longer times, when the conversion of fibrinogen to fibrin is complete. Thus, we conclude that the modulus of the fibrin network is increased both by calcium

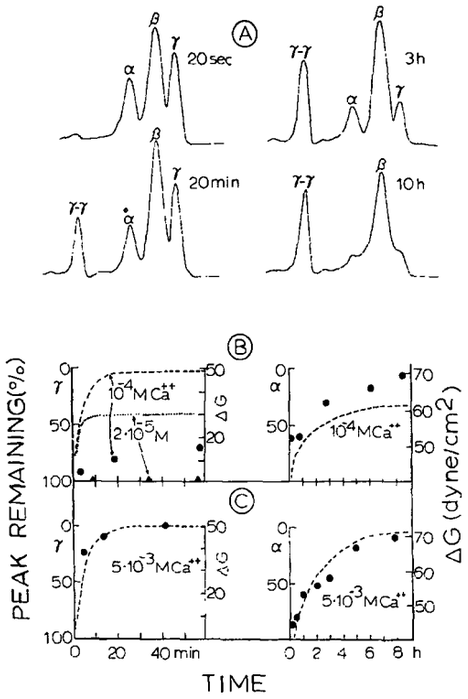

Fig. 2(A). Photometer tracings of 7% polyacrylamide gels containing sodium dodecyl sulfate and dithiothreitol (7) after electrophoresis of fibrinogen clotted with thrombin in the presence of active Factor XIII (10^{-4} M $CaCl_2$). (B). Points indicate fraction of non-crosslinked fibrin α- and γ-chains, versus time, as determined by SDS polyacrylamide gel electrophoresis for solutions containing 10^{-4} M and 2 x 10^{-5} M $CaCl_2$. α- and γ-chain quantities were determined by planimetry of gel scans taken at 570 nm of the Coomassie-blue stained gels; β-chain was used as internal standard. Curves show the difference in elastic modulus from that observed without added $CaCl_2$ (right hand scales). In the presence of 2 x 10^{-5} M $CaCl_2$ very little α-polymer is formed, and the modulus is almost the same at 8 h as at 1 h after clotting. (C). Same as for B, for solutions containing 5 x 10^{-3} M $CaCl_2$.

ion and by α-α crosslinks, but not by γ-γ crosslinks. In either case, the network stiffening may be the result of an increase in chain stiffness or of an increase in the concentration of network branchpoints. Above we noted an irreversible effect on the elastic modulus due to calcium removal by EDTA. Here we observe a reversible effect on the modulus, in line with the observation of other reversible effects of EDTA noted by several authors (11-15).

The rate of α-polymerization is slow and is not sensitive to calcium

concentration at moderate levels, while the rate of γ-dimerization is much more rapid and is sensitive to calcium concentration. These observations lead us to believe that 1) α-chain crosslinking by activated Factor XIII involves a slow reorganization of the fibrin network as the rate limiting process, either to give sideways thickening of strands or an increase in branchpoints, resulting in the observed network stiffening, but 2) γ-dimerization involves an end-to-end linking of fibrin molecules, which may occur as soon as the fibrin molecules associate (19,20), so that the rate is limited either by the rate of clotting or by the concentration of active enzyme causing the dimerization (Factor XIII-calcium complex), and therefore sensitive to the calcium concentration.

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References:

1. Ferry, J. D. (1948) Adv. Protein Chem. 4, 1-78.
2. Ferry, J. D., Miller, M. and Shulman, S. (1951) Arch. Biochem. 34, 424-436.
3. Laki, K. and Lorand, L. (1948) Science 108, 280.
4. Lorand, L. (1950) Nature 166, 694-695.
5. Lorand, L. and Konishi, K. (1964) Arch. Biochem. Biophys. 105, 58-67.
6. Hermans, J. (1965) J. Polymer Sci. A3, 1859-1868.
7. McDonagh, J., Messel, H., McDonagh, R. P., Murano, G. and Blombäck, B. (1972) Biochim. Biophys. Acta 257, 135-142.
8. Wagreich, H. and Tarlov, I. M. (1945) Arch. Biochem. 7, 345-352.
9. Ware, A. G., Guest, M. M. and Seegers, W. H. (1947) Arch. Biochem. 13, 231-236.
10. Lorand, L. (1972) Ann. N. Y. Acad. Sci. 202, 6-30.
11. Endres, G. F. and Scheraga, H. A. (1972) Arch. Biochem. Biophys. 153, 266-278.
12. Godal, H. C. (1960) Scand. J. Clin. Lab. Invest. 12, Suppl. 53, 1-20.
13. Godal, H. C. (1969) Scand. J. Clin. Lab. Invest. 24, 29-33.
14. Bithell, T. C. (1964) Biochem. J. 93, 431-439.
15. Blombäck, B., Blombäck, M., Laurent, T. C. and Pertoft, H. (1966) Biochim. Biophys. Acta 127, 560-562.
16. McKee, P. A., Mattock, P. and Hill, R. L. (1970) Proc. Natl. Acad. Sci. 66, 738-744.
17. McDonagh, R. P., McDonagh, J., Blombäck, M. and Blombäck, B. (1971) FEBS Letts. 14, 33-36.
18. Chen, R. and Doolittle, R. F. (1969) Proc. Natl. Acad. Sci. 63, 420-427.
19. Doolittle, R. F., Cassman, K. G., Chen, R., Sharp, J. J. and Wooding, G. L. (1972) Ann. N. Y. Acad. Sci. 202, 114-125.
20. McDonagh, R. P. Jr., McDonagh, J. and Blömback, B. (1972) Ann. N. Y. Acad. Sci. 202, 335-340.

SPINE Volume 27, Number 16S, pp S26–S31

Overview of the Biology of Lumbar Spine Fusion and Principles for Selecting a Bone Graft Substitute

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Study Design. Reviews were conducted.

Objectives. To review the biology of spine fusion healing, and to outline several fundamental principles required for the selection of a bone graft substitute.

Summary of Background Data. More than 200,000 spine fusions are performed each year in the United States. The success of this procedure is limited by morbidity from iliac crest bone graft harvest and a nonunion rate that ranges from 10% to 40%. In recent years, there has been an increased understanding of the biology of spine fusion healing. In addition, there has been a focus on finding suitable substitutes for autogenous iliac crest bone graft to promote spine fusion. The selection of a specific bone graft substitute can be a daunting task for the surgeon.

Methods. The available literature was reviewed and combined with the author's personal experience.

Results. A basic understanding of the biology of healing in different types of spine fusions and the differences between different categories of bone graft substitutes can help surgeons organize the graft selection process.

Conclusions. In general, purely osteoconductive substitutes are less effective in adult posterolateral spine fusions, but may be suitable in the anterior spine when it is rigidly immobilized. Osteoinductive substitutes are more likely to be successful as extenders, enhancers, or substitutes for posterolateral spine fusion. [Key words: animal models, biology, bone graft substitutes, bone morphogenetic proteins, gene expression, osteoconduction, osteoinduction, spine fusion] **Spine 2002;27:S26–S31**

It is estimated that more than 200,000 spine fusion procedures are performed each year in the United States. Posterolateral lumbar intertransverse process arthrodesis is the most common procedure performed, yet failure to achieve a solid bony union (nonunion) occurs in 10% to 40% of patients with single-level fusions, and more frequently when multiple levels are attempted.[15,19,25,26,30,61] This high rate of nonunions indicates that the physiologic, biologic, and molecular events crucial to this process are not adequately understood. A nonunion frequently leads to unsatisfactory resolution of clinical symptoms,[17,22,68] and usually results in greater medical costs and morbidity, as well as the need for additional surgeries.

From the Emory Spine Center, Decatur, Georgia.

Device Status/Drug Statement: The devices and drugs that are the subject of this manuscript are exempt from FDA or corresponding national regulations because BMP is not regulated by the FDA.

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The most common clinical approach for preventing nonunions has been the use of internal fixation (*e.g.*, hooks or screws with rods or plates). Although the use of internal fixation has decreased the number of nonunions, it has not eliminated the problem. Nonunions still occur in 10% to 15% of patients.[15,46,65,66] Clearly, other factors must be implicated, yet there is a paucity of knowledge regarding the biologic mechanisms involved.[20,43–45] Such knowledge would be helpful in devising strategies for nonunion prevention and allowing appropriate use of different bone graft substitutes for posterolateral spinal fusion.

The healing of a spine fusion is a multifactorial process, which makes it difficult to study in the clinical setting. The lack of reliable noninvasive techniques for assessing the success or failure of an arthrodesis further limits clinical studies.[16] Thus, an animal model is a practical solution for studying individual factors involved in this complex process.[57] Most models used before 1990 had limited utility for biologic studies for one or more of the following reasons: 1) the successful fusion rate was 100%, which is much higher than that seen clinically; 2) the animals were skeletally immature; 3) the arthrodesis performed was interfacet or interlaminar rather than intertransverse; or 4) the model destabilized the spine and resulted in 0% successful fusions without internal fixation.[35,51,54,55,67] The limitations of these earlier models led to the development of an intertransverse process arthrodesis model that was more clinically applicable to the human situation.[10] The major benefit of this type of model is that nonunions spontaneously occur at a rate comparable with that reported in humans.[10,57]

Biology of Spine Fusion

The rabbit lumbar intertransverse process arthrodesis model has been well characterized using autogenous iliac crest as the graft material. Mechanically solid fusions generally occur by the fourth postoperative week with an overall nonunion rate of 30% to 40%. Radiographic analysis has showed progressive remodeling of bone graft material with time, usually by 10 to 12 weeks, but as in humans, radiographs were accurate in assessing success or failure to attain solid fusion only 70% of the time. This model was further validated using control animals with omission of iliac bone graft placement or omission of the decortication step, which resulted in nonunion in all cases. These negative control animals showed that the surgical exposure alone did not automatically result in spinal fusion. This was a limitation of some previous models. Vascular injection studies indi-

cated that the primary blood supply to the fusion mass originated from the decorticated transverse processes, not the surrounding soft tissues.[63] The failure to achieve spine fusion in the absence of decortication emphasized the importance of extensive decortication of the posterolateral spine elements (lateral facet, pars interarticularis, transverse process) in providing bone marrow, vascularization, and osteoprogenitor cells to the fusion mass.

Histologic analysis of the rabbit transverse process fusion has showed three distinct and reproducible temporal phases of spine fusion healing.[12] Maturation of the spine fusion was most advanced at the ends of the fusion mass near the transverse processes ("outer" zone). A similar histologic progression occurred in the "central" zone, but was delayed in time. This central "lag effect," with a transient cartilaginous area, may explain why many nonunions occur in the central zone of a fusion mass.

A unique temporal and spatial pattern of osteoblast-related gene expression was observed in an RT/PCR analysis of RNA from the different zones of the fusion mass. A lag effect in gene expression that correlated with the previously observed lag effect in the histologic healing sequence was noted in the central zone as compared with the outer zones of the fusion. As with osteocalcin expression, the peak expression of all genes measured was seen in the central zone 1 to 2 weeks later than the peak in the outer zone. This is consistent with the peripheral to central healing pattern observed histologically for fusions using autogenous bone graft.

Expression of the mRNA of several bone morphogenetic proteins (BMPs) also was studied. In the peripheral zones, BMP-2 mRNA expression was increased during weeks 2 through 6, with peak expression in weeks 3 and 4 (40-fold increase). Whereas BMP-6 in the outer zones had a first peak on day 2 (54-fold) and a second peak (100-fold) during week 5, BMP-6 in the central zone showed an initial peak on day 2 (34-fold), but did not demonstrate the later peak. These findings suggest specific time patterns of expression and probably unique roles for each of the various BMPs during spine fusion. It appears that BMP-6 is somewhat unique in that its mRNA levels demonstrated the earliest peak and greatest relative increase of the BMPs studied. The lower level of BMP-6 expression in the central zone of the fusion mass is correlated with the delayed timing and smaller amount of bone formation in the central zone of the fusion. Thus, the predilection for nonunion in the central zone also is apparent at a molecular biologic level.[50]

■ Models of Spine Nonunion

There are several models that can produce a higher rate of nonunion. Implanting only 50% of the normal volume of autograft lowered the fusion rate from 70% to 33% in the rabbit model.[39] Excessive spine motion (produced by lifting the rabbits out of their cage daily after surgery) decreased the fusion success rate from 58% to 14% ($P = 0.04$).[21] Moderate systemic nicotine exposure (equaling 1 to 2 packs of cigarettes per day) significantly decreased the rate of successful spine fusion from 56% to 0% ($P = 0.02$) in rabbits.[60] These results highlight the importance of recognizing that each healing environment has unique features, and that bone graft substitutes must be tested in the specific healing environments anticipated to predict efficacy. Ketorolac, commonly used as an intravenous analgesic during the postoperative period, facilitated the fourth nonunion model in the current study. Some nonsteroidal antiinflammatory drugs (NSAIDs) have been shown to inhibit fracture repair.[31,56,62,64] The current author determined that a standard dose (4 mg/kg/day) for 7 days caused a decrease in the successful fusion rate from 75% in the control group to 35% ($P < 0.04$) in the rabbits treated with ketorolac.[40]

In summary, the biology of lumbar spine fusion healing in rabbits has been well characterized with autograft, and important mechanical, vascular, cellular, and molecular influences have been identified. Similar detailed understanding is needed for anterior interbody fusion healing, although this location may be less challenging because of increased bony surface area and compression loading. Bone graft quality, quantity, and host bed vascularity (decortication) are critical and must be assessed carefully by the surgeon. The choice of graft substitutes must be individualized on the basis of the aforementioned factors and the presence of potential local or systemic inhibitory factors.

■ Augmentation of Bone Healing in the Spine

It is estimated that approximately 500,000 bone graft procedures are performed in the United States each year. Of these bone-grafting procedures, the vast majority (≈50%) are spine fusions, followed by general orthopedic procedures and craniomaxillofacial procedures. This potentially represents a $0.4 to $2 billion per year market for the use of bone repair enhancers or bone graft substitutes (DataMonitor, personal communication).

The most common reason for performing a spinal arthrodesis is either instability (excessive motion) of a spine segment or a deformity that is at risk for progression. The vast majority of fusions are performed to treat degenerative disorders, and the most common site is the lumbar spine. Although spinal fusion is commonly attempted, nonunion is reported to occur in 5% to 45% of patients.[42,52,61,66] Although this may be a clinically disturbing statistic, it makes spine fusion an ideal venue for testing bone augmentation devices. It is far easier to show an improvement in the healing of spine fusion than in the healing of fracture repair, in which the normal healing is quite good. Another rationale for testing bone augmentation in spine fusion is the reality that there is frequently an inadequate supply of autogenous bone graft for performing multilevel spinal arthrodesis. In addition, the morbidity of iliac bone graft harvest is reported to be as high as 30%, with the most frequent complications including chronic donor site pain, infection, fracture, hematoma, and increased operative time and costs.[1,5,23,34,58] The current author estimated that the cost of an iliac crest bone graft harvest is approximately $1500 to $2500.

Currently, there are several competitive strategies either to augment healing or to replace autogenous bone

Table 1. Potential Roles for Bone Graft Materials

Role	Amount of ABG	Graft Volume	Healing Success
Extender	Usual or less	More or same	Same as ABG
Enhancer	Usual or less	More or same	Better than ABG
Substitute	None	Same or more	Same or better than ABG

ABG = autogenous bone graft.

graft in the spine.[9] The most commonly used strategy for healing enhancement is that of rigid internal fixation. The fixation devices range in cost from $3000 to $6000, and are reported to increase the efficacy or the successful fusion rate by approximately 10%. Biophysical stimulation is another strategy that has been pursued in spine fusion for some time, but without good clinical data to support its use. Currently, the costs estimated for electrical stimulation by either direct current or pulsed electromagnetic fields is approximately $4000 to $5000, and although prospective randomized blinded trials have not yet been completed, the maximum benefit in terms of an increased successful fusion rate is estimated to be approximately 10%.[47,48] Low-intensity ultrasound also has been proposed recently for accelerating the healing of long bone fractures, and some experimental evidence in animal models shows that this may also be a viable enhancement strategy for spine fusion.[27,29]

Currently, a variety of potential bone graft extenders is available. Demineralized bone matrix (DBM) in a variety of forms, each with variable degrees of osteoinductivity, is available through numerous sources. In general, the DBM cost for augmenting a one-level spinal fusion may be as high as $1100, and the current data support the use of DBM as a bone graft extender only and not as a bone graft enhancer or substitute.[49] Similarly, various ceramics such as natural coral (calcium carbonate), coralline hydroxyapatite, or composites such as hydroxyapatite-tricalcium phosphate have been used on a limited basis. Although clinical studies are lacking, limited animal data suggest that these ceramics may be viable as bone graft extenders when mixed with autogenous bone graft, but that they are unlikely to serve as stand-alone bone graft substitutes.[11] Thus, the need for improved bone repair and augmentation in the area of spine fusion is evident. The remainder of this article focuses on issues pertaining to the selection of an acceptable bone graft substitute for patients undergoing various types of spine fusion. For the purposes of this discussion, a graft extender is considered to be a material that allows the use of less autogenous bone graft with the same end result, or one that allows a given amount of autogenous bone to be stretched over a greater area with the same success rate (Table 1). A bone graft enhancer is a device that when added to autogenous bone graft, increases the successful healing rate of autogenous bone graft, using either the usual amount of graft or a smaller amount of bone graft. Finally, a bone graft substitute is a material that may be used entirely in place of autogenous bone graft to achieve the same or a better fusion success rate.

Because prospective randomized clinical trials that isolate the efficacy of individual bone graft substitutes in the spine do not exist, it can be challenging to garner information from the published animal and clinical studies. Several overriding principles should be kept in mind. The mechanism and timing of bone healing may vary considerably depending on the region of the spine. The anterior or middle column of the spine is primarily cancellous bone and load bearing, whereas the posterior column of the spine has a greater combination of cortical bone and a submuscular healing environment frequently under tension. Thus, there is considerable variation in the type of healing and speed of healing because of these biologic and biomechanical differences in healing environment. The three primary locations are the anterior interbody, the intertransverse process, and the interlaminar–facet joint region. As a result of these regional differences, the dosage and the ideal delivery vehicle for bone graft substitutes may differ by location. Again, it cannot be overemphasized that the results of healing for bone graft substitutes or augmentation devices in one region of the spine cannot necessarily be extrapolated for other regions.

In addition to the region of the spine as a cause for differences in healing, the specific patient diagnosis may give rise to variation in healing success. One example is lower lumbar spinal fusion for degenerative spondylolisthesis. This may be associated with increased motion at the intervertebral disc space to be fused, which decreases the rate of successful fusion. Alternatively, a patient with degenerative lumbar disc disease and disc collapse may actually have decreased disc motion, which may result in an increased likelihood of obtaining a solid fusion. The risks of neurologic complication, which is the most serious complication resulting from spine surgery, also is dependent on location. Cervical and thoracic spine fusions, particularly in the anterior column, carry some risks of spinal cord injury. In contrast, the structure at risk in the lumbar spine generally is the cauda equina, which is a more tolerant neurologic structure.

Another issue unique to spine fusion is the increased difficulty in the actual assessment of intervention efficacy. It is not always obvious when a solid fusion has been obtained, as opposed to a pseudarthrosis or nonunion. Unfortunately, the vast majority of noninvasive evaluation methods are inaccurate. It is reported that plain radiographs carry an accuracy no greater than 70% for predicting whether a spine fusion is solid.[16,18,28,52] Although the current author believes empirically that CT scanning is a more accurate method, there are no good data available to qualify this increased accuracy in the assessment of spine fusion. Another confounding variable involves the frequent dissociation between measurement of an augmentation device's success (*i.e.*, solid spine fusion) and clinical outcome (*e.g.*, decreased pain or increased function). This dissociation is particularly germane to spine fusion, and is substantiated further by the lack of clinical consensus as to the appropriate indications for spine fusion.[24]

Selecting a Bone Graft Substitute: The Burden of Proof

The donor site morbidity, limited supply, and imperfect success rate of autogenous bone graft has led to the commercialization of a variety of bone graft substitutes. These graft alternatives have undergone varying degrees of regulatory scrutiny, so their true safety and effectiveness in patients may not be known before their use by orthopedic surgeons. Moreover, given the different categories of bone graft substitutes and the seemingly similar products within each category, product differentiation has become increasingly varied for the practicing spine surgeon. This section is intended to suggest a framework of principles for considering the burden of proof necessary to allow clinicians to make reasonable choices when deciding on osteoinductive bone graft substitutes.

Osteoconductive substitutes may be used as scaffolds to deliver bone growth factors. These materials facilitators for bone formation, but do not contain growth factors that can attract osteoblast precursor cells and initiate osteoblast differentiation. Osteoconductive materials include collagen, ceramics, polymers, and coral, whereas osteoinductive substitutes contain one or more growth factors such as bone morphogenetic proteins (BMPs) capable of independently attracting precursor cells and inducing new bone formation. Whereas numerous growth factors may be involved in new bone formation including platelet derived growth factor (PDGF), transforming growth factor-beta (TGF-β), and vascular endothelial growth factor (VEGF), evidence suggests that only the BMPs are capable of initiating the entire process of new bone formation.[6,7,9,13,36] Lower doses of the BMPs are contained in demineralized bone matrix. They can be found in higher doses as recombinant (genetically engineered) proteins or purified extracts from bone. These materials are being studied in prospective trials and should soon be available for selected uses.

For understanding the burden of proof, the first principle to consider is that different healing environments (*e.g.*, metaphyseal defect, long bone fracture, interbody spine fusion, and posterolateral spine fusion) have increasing levels of difficulty in forming new bone. For example, a metaphyseal defect permits the successful use of many purely osteoconductive materials. In contrast, a posterolateral spine fusion environment generally does not tolerate the use of purely osteoconductive materials as stand-alone substitutes, and only sometimes permits their use as bone graft extenders. Thus, validation of any bone graft substitute in one clinical anatomic site may not be predictive of its performance in another location. Accordingly, surgeons must be very careful in using graft substitutes that have not been tested definitively in the particular healing environment wherein they are about to use it. Most of the osteoconductive substitutes used currently in the spine have been validated only in nonspine locations.

The second principle to consider is the burden of proof required from preclinical studies to justify the use of an osteoinductive graft material or the choice of one brand over another. An osteoinductive material, by definition, must contain one or more growth factors including BMPs that can induce *de novo* ectopic bone formation through differentiation of progenitor cells into osteoblasts. Although not commonly recognized, evidence clearly suggests that it is much harder to make bone in humans than in cell culture or rodent models.[8] It is generally accepted that in lower vertebrates, bone heals more readily than in humans. Some osteoinductive materials (*e.g.*, DBM and rhBMP-2) have been successful in lower vertebrates, but have failed to achieve the same result in primates.[2–4] Findings also have shown the rhesus monkey also to be an acceptable model for human bone remodeling and osteoporosis.[32,33,37,38,53,59]

Only human trials can determine the efficacy of osteoinductive materials in humans. However, a hierarchy of testing credibility exists, ranging from cell culture assays of bone markers and the rat ectopic bone induction assay, as the least predictable tests, to rabbit ulnar defect, rabbit

Table 2. Burden of Proof for Osteoinductive Bone Graft Materials for Spine Fusion

Model	Burden of Proof
Cell culture	Establish that a protein is osteoinductive if bone nodules are the endpoint. If surrogate endpoints are used (alkaline phosphatase or osteocalcin production, these assays are less reliable.
Rat ectopic assay	Establish feasibility of *in vivo* ectopic bone induction. Because rats heal in only 2–3 weeks, issues of premature carrier resorption or compressibility may not be detected in rapidly resorbing carriers. Also, slower resorbing carriers may not form as much bone because the carrier is still occupying much volume in the implant.
Rodent spine fusion	Establish feasibility of successful spine fusion in environment with motion and/or loading. Healing time frame is longer than rat, but still only 4–8 weeks and thus same issues to do with carrier resorption time apply. Useful if detailed biomechanical testing is required.
Large animal model	Establish efficacy in spine fusion model with internal fixation if needed. Internal fixation not practical in most rodent models. Larger animals do not necessarily present a more challenging healing environment and are not more predictive of responses in humans compared with rodents. Useful if detailed biomechanical testing is required.
Nonhuman primates	Nonhuman primates, especially rhesus monkeys, have similar osteoinduction responsiveness, healing time, and carrier resorption behavior to humans. Osteoinductive growth factors require 10–15-fold higher concentrations to achieve comparable results in primates as seen in rodents. Successful doses in rhesus monkeys have translated well into required doses for BMPs in human clinical trials.
Human clinical trials	The ultimate proof lies in a human clinical trial. Doses, carrier requirements, and the need for supplemental internal fixation may vary based on anatomic location in the spine, patient diagnosis, and presence of healing deterrants (smoking, steroids, diabetes, etc).

spine fusion, and large animal long bone–spine fusion, which have intermediate predictability of efficacy in humans (Table 2). Nonhuman primate long bone, interbody spine, and posterolateral spine fusion models offer the highest challenge and therefore the highest predictability of success in humans.[14,41] It is most important to understand that failure of an osteoinductive material to induce bone in any of these progressively challenging models usually means that it will fail at all higher levels. Unfortunately, successful bone induction at any level does not infer success at the next most stringent level. Only testing at the next level can answer that question. Thus, when differentiating between brands of similar osteoinductive products that have not been validated in human clinical trials, the surgeon should strongly consider choosing the product that satisfied the highest burden of proof in preclinical studies within the aforementioned hierarchy.

The third principle involving the burden of proof specifically pertains to products not currently subject to high levels of regulation such as demineralized bone matrix (DBM) and platelet gels containing "autologous growth factors." Such products are considered to involve minimal manipulation of cells or tissue, and thus are regulated as tissue rather than devices. As a result, no standardized burden of proof level for safety or effectiveness required is before these products are marketed and used in human patients. Although it may be true that such products meet the technical definition of "minimal manipulation," thereby escaping strict regulation, DBM and "autologous growth factor" platelet concentrates are being marketed and used for their biologic (osteoinductive) effects, not as simple tissue transplants or means of augmentation. Consequently, there is a risk that products or certain brands of products may not produce the expected result in humans when there has been little or no animal testing in the relevant and more challenging models. Many investigators in this field believe that minimal standards demonstrating some level of osteoinductivity (bone induction) should be developed for such products. If surgeons do not demand this soon, or if the manufacturers do not institute this in the near future, these products may risk being more highly regulated by the FDA, making them less accessible to surgeons and patients.

■ Key Points

- A basic understanding of the biology of healing in different types of spine fusions and the differences between different categories of bone graft substitutes can help surgeons organize the graft selection process.
- In general, purely osteoconductive substitutes are less effective in adult posterolateral spine fusions, but may be suitable in the anterior spine when it is rigidly immobilized.
- Osteoinductive substitutes are more likely to be successful as extenders, enhancers, or substitutes for posterolateral spine fusion.

References

1. Arrington ED, Smith WJ, Chambers HG, et al. Complications of iliac crest bone graft harvesting. Clin Orthop 1996;329:300–9.
2. Aspenberg P, Lohmander LS, Thorngren K-L. Failure of bone induction by bone matix in adult monkeys. J Bone Joint Surg Br 1988;70:625–7.
3. Aspenberg P, Turek T. BMP-2 for intramuscular bone induction: Effect in squirrel monkeys is dependent on implantation site. Acta Orthop Scand 1996;67:3–6.
4. Aspenberg P, Wang E, Thorngren K-G. Bone morphogenetic protein induces bone in the squirrel monkey, but bone matrix does not. Acta Orthop Scand 1992;63:619–22.
5. Banwart JC, Asher MA, Hassanein RS. Iliac crest bone graft harvest donor site morbidity: A statistical evaluation. Spine 1995;20:1055–60.
6. Boden SD. Bioactive factors for bone tissue engineering. Clin Orthop 1999; 367(Suppl):S84–94.
7. Boden SD. Clinical application of the BMPs. J Bone Joint Surg Am 2001; 83(Suppl 1):S161.
8. Boden SD, McCuaig K, Hair G, et al. Differential effects and glucocorticoid potentiation of bone morphogenetic protein action during rat osteoblast differentiation *in vitro*. Endocrinology 1996;137:3401–7.
9. Boden SD, Schimandle JH. Biologic enhancement of spinal fusion. Spine 1995;20:113S–23S.
10. Boden SD, Schimandle JH, Hutton WC. An experimental lumbar intertransverse process spinal fusion model: Radiographic, histologic, and biomechanical healing characteristics. Spine 1995;20:412–20.
11. Boden SD, Schimandle JH, Hutton WC, et al. *In vivo* evaluation of a resorbable osteoinductive composite as a graft substitute for lumbar spinal fusion. J Spinal Disord 1997;10:1–11.
12. Boden SD, Schimandle JH, Hutton WC, et al. The use of an osteoinductive growth factor for lumbar spinal fusion: Part I. The biology of spinal fusion. 1995 Volvo Award in Basic Sciences. Spine 1995;20:2626–32.
13. Boden SD, Sumner DR. Biologic factors affecting spinal fusion and bone regeneration. Spine 1995;20:102S–12S.
14. Boden SD, Zdeblick TA, Sandhu HS, et al. The use of rhBMP-2 in interbody fusion cages: Definitive evidence of osteoinduction in humans: A preliminary report. Spine 2000;25:376–81.
15. Bridwell KH, Sedgewick TA, O'Brien MF, et al. The role of fusion and instrumentation in the treatment of degenerative spondylolisthesis with spinal stenosis. J Spinal Disord 1993;6:461–72.
16. Brodsky AE, Kovalsky ES, Khalil MA. Correlation of radiographic assessment of lumbar spine fusions with surgical exploration. Spine 1991;16: S261–5.
17. Conaty JP, Mongan ES. Cervical fusion in rheumatoid arthritis. J Bone Joint Surg Am 1981;63:1218–27.
18. Dawson EG, Clader TJ, Bassett LW. A comparison of different methods used to diagnose pseudarthrosis following posterior spinal fusion for scoliosis. J Bone Joint Surg Am 1985;67:1153–9.
19. DePalma AF, Rothman RH. The nature of pseudarthrosis. Clin Orthop 1968;59:113–18.
20. Farey ID, McAfee PC, Gurr KR, et al. Quantitative histologic study of the influence of spinal instrumentation on lumbar fusions: A canine model. J Orthop Res 1989;7:709–22.
21. Feiertag MF, Boden SD, Schimandle JH, et al. A rabbit model for nonunion of lumbar intertransverse process spine arthrodesis. Spine 1996;21:27–31.
22. Ferlic DC, Clayton ML, Leidholt JD, et al. Surgical treatment of the symptomatic unstable cervical spine in rheumatoid arthritis. J Bone Joint Surg Am 1975;57:349–54.
23. Fernyhough JC, Schimandle JH, Weigel MC, et al. Chronic donor site pain complicating bone graft harvesting from the posterior iliac crest for spinal fusion. Spine 1992;17:1474–80.
24. Fischgrund JS, Mackay M, Herkowitz HN, et al. Degenerative lumbar spondylolisthesis with spinal stenosis: A prospective, randomized study comparing decompressive laminectomy and arthrodesis with and without spinal instrumentation. Spine 1997;22:2807–12.
25. Fischgrund JS, Mackay M, Herkowitz HN, et al. Volvo Award winner in clinical studies: Degenerative lumbar spondylolisthesis with spinal stenosis: A prospective, randomized study comparing decompressive laminectomy and arthrodesis with and without spinal instrumentation. Spine 1997;1997; 22:2807–12.
26. France JC, Yaszemski MJ, Lauerman WC, et al. A randomized prospective study of posterolateral lumbar fusion: Outcomes with and without pedicle screw instrumentation. Spine 1999;24:553–60.
27. Glazer PA, Heilmann MR, Lotz JC, et al. Use of electromagnetic fields in a spinal fusion: A rabbit model. Spine 1997;22:2351–6.
28. Hannon KM, Wetta WJ. Failure of technetium bone scanning to detect pseudarthrosis in spinal fusion for scoliosis. Clin Orthop 1977;123:42–4.

29. Heckman JD, Ryaby JP, McCabe J, et al. Acceleration of tibial fracture healing by noninvasive, low-intensity pulsed ultrasound. J Bone Joint Surg Am 1994;76:26–34.
30. Herkowitz HN, Kurz LT. Degenerative lumbar spondylolisthesis with spinal stenosis: A prospective study comparing decompression with decompression and intertransverse process arthrodesis. J Bone Joint Surg Am 1991;73: 802–8.
31. Keller J, Bunger C, Andreassen TT, et al. Bone repair inhibited by indomethacin: Effects on bone metabolism and strength of rabbit osteotomies. Acta Orthop Scand 1987;58:379–83.
32. King FA, Yarbrough CJ. Medical and behavioral benefits from primate research. Physiologist 1985;28:75–87.
33. King FA, Yarbrough CJ, Anderson DC, et al. Primates. Science 1988;240: 1475–82.
34. Laurie SWS, Kaban LB, Mulliken JB, et al. Donor-site morbidity after harvesting rib and iliac bone. Plast Reconstr Surg 1984;73:933–8.
35. Lindholm TS, Ragni P, Lindholm TC. Response of bone marrow stroma cells to demineralized cortical bone matrix in experimental spinal fusion in rabbits. Clin Orthop 1988;230:296–302.
36. Ludwig SC, Boden SD. Osteoinductive bone graft substitutes for spinal fusion: A basic science summary. Orthop Clin North Am 1999;30:635–45.
37. Mann DR, Gould KG, Collins DC. A potential primate model for bone loss resulting from medical oophorectomy or menopause. J Clin Endocrinol Metab 1990;71:105–10.
38. Mann DR, Rudman GG, Akinbami MA, et al. Preservation of bone mass in hypogonadal female monkeys with recombinant human growth hormone administration. J Clin Endocrinol Metab 1992;74:1263–9.
39. Martin G, Boden SD, Morone MA, et al. New formulations of demineralized bone matrix as a more effective graft alternative in experimental posterolateral lumbar spine arthrodesis. Spine 1999;24:637–45.
40. Martin G, Boden SD, Titus L. Recombinant human bone morphogenetic protein-2 reverses the inhibitory effect of ketorolac, a nonsteroidal antiinflammatory drug (NSAID) on posterolateral lumbar intertransverse process spine fusion. Spine 1999;24:2188–94.
41. Martin GJ, Boden SD, Morone MA, et al. Posterolateral intertransverse process spinal fusion arthrodesis with rhBMP-2 in a nonhuman primate: Important lessons learned regarding dose, carrier, and safety. J Spinal Disord 1999;12:179–86.
42. May VR, Mauck WR. Exploration of the spine for pseudarthrosis following spinal fusion in the treatment of scoliosis. Clin Orthop 1967;53:115–22.
43. McAfee PC, Farey ID, Sutterlin CE, et al. Device-related osteoporosis with spinal instrumentation. Spine 1989;14:919–26.
44. McAfee PC, Farey ID, Sutterlin CE, et al. The effect of spinal implant rigidity on vertebral bone density: A canine model. Spine 1991;16:S190–7.
45. McAfee PC, Regan JJ, Farey ID, et al. The biomechanical and histomorphometric properties of anterior lumbar fusions: A canine model. J Spinal Disord 1988;1:101–10.
46. McGuire RA, Amundson GM. The use of primary internal fixation in spondylolisthesis. Spine 1993;18:1662–72.
47. Meril AJ. Direct current stimulation of allograft in anterior and posterior lumbar interbody fusions. Spine 1994;19:2393–8.
48. Mooney V. A randomized double-blind prospective study of the efficacy of pulsed electromagnetic fields for interbody lumbar fusions. Spine 1990;15: 708–12.
49. Morone MA, Boden SD. Experimental posterolateral lumbar spinal fusion with a demineralized bone matrix gel. Spine 1998;23:159–67.
50. Morone MA, Boden SD, Martin G, et al. Gene expression during autograft lumbar spine fusion and the effect of bone morphogenetic protein-2. Clin Orthop 1998;351:252–65.
51. Muschler GF, Huber B, Ullman T, et al. Evaluation of bone grafting materials in a new canine segmental spinal fusion model. J Orthop Res 1993;11: 514–24.
52. O'Beirne J, O'Neill D, Gallagher J, et al. Spinal fusion for back pain: A clinical and radiological review. J Spinal Disord 1992;5:32–8.
53. Pope NS, Gould KG, Anderson DC, et al. Effects of age and sex on bone density in the rhesus monkey. Bone 1989;10:109–12.
54. Ragni PC, Lindholm TS. Bone formation and static changes in the thoracic spine at uni- or bilateral experimental spondylodesis with demineralized bone matrix (DBM). Ital J Orthop Traumatol 1989;15:237–52.
55. Ragni PC, Lindholm TS, Lindholm TC. Vertebral fusion dynamics in the thoracic and lumbar spine induced by allogenic demineralized bone matrix combined with autogenous bone marrow: An experimental study in rabbits. Ital J Orthop Traumatol 1987;13:241–51.
56. Ro J, Sudmann E, Marton PF. Effect of indomethacin on fracture healing in rats. Acta Orthop Scand 1976;47:588–99.
57. Schimandle JH, Boden SD. The use of animal models to study spinal fusion. Spine 1994;19:1998–2006.
58. Schnee CL, Freese A, Weil RJ, et al. Analysis of harvest morbidity and radiographic outcome using autograft for anterior cervical fusion. Spine 1997;22:2222–7.
59. Schnitzler CM, Ripamonti U, Mesquita JM. Histomorphometry of iliac crest trabecular bone in adult male baboons in captivity. Calcif Tissue Int 1993; 52:447–54.
60. Silcox DH, Daftari T, Boden SD, et al. The effect of nicotine on spinal fusion. Spine 1995;20:1549–53.
61. Steinmann JC, Herkowitz HN. Pseudarthrosis of the spine. Clin Orthop 1992;284:80–90.
62. Sudmann E, Dregelid E, Bessesen A, et al. Inhibition of fracture healing by indomethacin in rats. Eur J Clin Invest 1979;9:333–9.
63. Toribatake Y, Hutton WC, Boden SD, et al. Revascularization of the fusion mass in a posterolateral intertransverse process fusion. Spine 1998;23:1149–54.
64. Tornkvist H, Lindholm S. Effect of ibuprofen on mass and composition of fracture callus and bone. Scand J Rheumatol 1980;9:167–71.
65. West JL III, Bradford DS, Ogilvie JW. Results of spinal arthrodesis with pedicle screw plate fixation. J Bone Joint Surg Am 1991;73:1179–84.
66. Zdeblick TA. A prospective, randomized study of lumbar fusion: Preliminary results. Spine 1993;18:983–91.
67. Zerwekh JE, Kourosh S, Scheinberg R, et al. Fibrillar collagen–biphasic calcium phosphate composite as a bone graft substitute for spinal fusion. J Orthop Res 1992;10:562–72.
68. Zoma A, Sturrock RD, Fisher WD, et al. Surgical stabilisation of the rheumatoid cervical spine: A review of indications and results. J Bone Joint Surg Br 1987;49:8–12.

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10	50005-01-01 SOLICITUDES	1607 REIVINDICACION UNITARIA ADICIONAL A LAS 10 INICICIALES	30.000.00	300.000.00
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