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Referencia: Solicitud de Patente de Invención PCT denominada
**“ERITROPOYETINA HUMANA CON DISMINUCIÓN DE SU
ACTIVIDAD ERITROPOYETICA, CON MUTACIONES QUE
GENERAN SITIOS DE GLICOSILACIÓN Y PROCESO
PARA SU OBTENCIÓN”**

Asunto: Respuesta al oficio No. 2680 de 15 de febrero de 2024

Expediente No. NC2021/0004447

Juan Carlos Cuesta Quintero, mayor de edad, identificado como aparece al pie de mi firma, obrando en mi calidad de apoderado de **UNIVERSIDAD NACIONAL DEL LITORAL, CONSEJO NACIONAL DE INVESTIGACIONES CIENTIFICAS Y TECNICAS (CONICET), UNIVERSIDAD NACIONAL DE GENERAL SAN MARTÍN** doy respuesta al Oficio No. 2680 de 15 de febrero de 2024, bajo los siguientes términos:



1. Excepción de Patentabilidad

El Examinador manifiesta que el objeto de las reivindicaciones 15 y 16 no es patentable, al no referir una proporción o concentración de un principio activo en términos de porcentaje, molaridad o similares; sino a una cantidad requerida para obtener un efecto terapéutico. En consecuencia y para dar por superada la objeción, se han modificado estas reivindicaciones indicando ahora la concentración de la eritropoyetina humana modificada, como se muestra a continuación:

“Una composición farmacéutica caracterizado porque comprende una concentración desde 50 hasta 400 ng/ml de la eritropoyetina humana modificada de la reivindicación 1...”

Por lo tanto, la objeción ha sido superada y en este sentido las anteriores reivindicaciones 15 y 16, ahora reivindicaciones 11 y 12, son patentables.

2. Reivindicaciones relativas a un uso

El Examinador indica que la reivindicación 17 no se considera patentable, ya que, a pesar de que su preámbulo se refiere a la Eritropoyetina humana modificada de la reivindicación 1, no se incluye ninguna característica técnica esencial adicional a las ya referidas en las reivindicaciones precedentes.

En ese sentido, el Solicitante ha decidido eliminar la anterior reivindicación 17 y con esto dar por superada la objeción.



3. De la solicitud de patente

3.1. Título

El titular de la presente invención presenta la siguiente propuesta para enmendar el título tal como sigue:

**“ERITROPOYETINA HUMANA CON DISMINUCIÓN DE SU ACTIVIDAD
ERITROPOYETICA, CON MUTACIONES QUE GENERAN SITIOS DE GLICOSILACIÓN
Y PROCESO PARA SU OBTENCIÓN”.**

3.2. Reivindicaciones

En respuesta a lo indicado por el Examinador referente a la falta de concisión del capítulo al comprender múltiples reivindicaciones independientes, el Solicitante ha modificado las reivindicaciones de la 7 a la 14 incluyendo la dependencia directa e indirecta a la reivindicación 1, tal como se propone en el pliego adjunto.

Adicionalmente y para dar claridad a la reivindicación 1, el Solicitante ha modificado la reivindicación 1 y en esta ahora sólo se reivindican las tres secuencias correspondientes a las eritropoyetinas mutadas que han sido objeto de los ejemplos de aplicación presentados en la solicitud original.

En lo que respecta a las objeciones a las reivindicaciones 1, 3 y 4 que considera el Examinador como resultados a alcanzar, y que refiere la baja acción eritropoyética, respetuosamente se difiere de las apreciaciones, ya que se hace referencia a la actividad eritropoyética menor al 0,5 % en el exordio de la primera reivindicación expresando una característica que define el campo tecnológico, no pretendiendo caracterizar la invención



con tal efecto técnico. Adicionalmente, se han eliminado, según las recomendaciones del Sr. Examinador las reivindicaciones originales 3 y 4.

En lo referente a la reivindicación y según lo sugerido, se ha eliminado la reivindicación 2. Asimismo, se han modificado la dependencia de la reivindicación 5 y eliminado la reivindicación 6.

La reivindicación 12, ahora reivindicación 8, ha sido modificada y ahora depende de la reivindicación 6.

Las reivindicaciones de proceso (18 original en adelante) han sido enmendadas y ahora reflejan el proceso para obtener las muteínas de la invención, caracterizadas en la enmendada reivindicación 1. Por lo que se entiende superada la objeción.

La reivindicación 20 ha sido enmendada definiendo los vectores utilizados.

Con todas las modificaciones y argumentos presentados, se han dado por superadas las objeciones presentadas por el Examinador.

3.3. Descripción

En respuesta a lo expresado por el Examinador en relación con las supuestas inconsistencias de la nomenclatura se dice lo siguiente:

Las proteínas de uso terapéutico producidas por células eucariotas son proteínas de secreción. Por lo tanto, son incorporadas de manera cotraduccional al retículo



endoplasmático (RE), y viajan a través del aparato de Golgi y las vesículas secretorias hasta llegar al exterior de la célula.

La síntesis de las proteínas de secreción comienza en los ribosomas libres, localizados en el citosol. Luego de la expresión de un péptido señal de localización en el RE, de 16 a 30 residuos, la proteína de reconocimiento de señal (SRP, de su sigla en inglés signal recognition particle) interacciona con el complejo ARN ribosoma proteína, interrumpiendo la traducción. De este modo, todo el complejo es dirigido hacia el RE. La SRP interacciona con su receptor, presente en la membrana del RE, desencadenando la translocación de la proteína naciente al interior del sistema de endomembranas a través de un conjunto de proteínas denominado translocón. Una vez en el lumen del RE, el péptido señal es clivado por la peptidasa señal presente en dicho compartimiento y la síntesis de la proteína continúa su curso hacia el interior del sistema de endomembranas.

Por lo anteriormente expresado, el péptido señal es una condición indispensable para que la proteína pueda secretarse al medio de producción en su estado final maduro pero su secuencia es escindida antes de que ello ocurra. De este modo, la inclusión del péptido señal es usual en las comunicaciones científicas en la materia, aunque no forma parte de la proteína madura. En general el péptido señal no solamente es útil para la secreción, sino que puede jugar un rol en la adquisición de la proteína nativa o madura. Por tal motivo es que en la solicitud original la expresión de la secuencia comprende el péptido señal. El péptido señal utilizado fue descrito en la bibliografía científica en el año 1985: Proc Natl Acad Sci U S A. 1985 Nov;82(22):7580-4. doi: 10.1073/pnas.82.22.7580. O también en Biotecnología Aplicada, Vol 8, No.3 pág:326-334 (1991).

Sin embargo, coincidimos en que para una mayor claridad es posible expresar el objeto principal de la presente invención en una secuencia que no comprenda al péptido señal. Es por esta razón que tomando las observaciones del Sr. Examinador, que se agradecen,



presentamos un nuevo listado de secuencias en que se han eliminado los primeros 27 AA del péptido señal. Asimismo, se elimina el último aminoácido (Arg166) dado que la secuencia madura contiene 165 aminoácidos y finaliza con el residuo Asp165. Es decir, que la secuencia inmadura de 193 aminoácidos además de sufrir una modificación cotraslacional como la pérdida del péptido señal de 27 aminoácidos, sufre una modificación postraslacional correspondiente a la pérdida del residuo arginina de la posición 166. Todas estas modificaciones llevan a una hEPO presente en circulación sanguínea de 165 aminoácidos (Ver descripción de la secuencia final de aminoácidos de la hEPO en la publicación científica del año 1992, donde claramente se describe la eliminación del aminoácido 166 para dar lugar a una hEPO madura de 165 aminoácidos: *Physiol Rev.* 1992 Apr;72(2):449-89. doi: 10.1152/physrev.1992.72.2.449.)

El péptido señal, de 27 aminoácidos y cuya escisión da lugar a la molécula de hEPO madura de 165 aminoácidos, tiene la secuencia aminoacídica: mgvhecpawlwlslslplglpvlg). La memoria descriptiva de la presente invención tal como ha sido presentada originalmente contempló las modificaciones indicadas sobre la secuencia de EPO madura (sólo secuencia de aminoácidos de EPO) con la numeración o nomenclatura establecida sobre ella, tal como se encuentra en la bibliografía y como es utilizada por los expertos en la materia. Se incorporó al cuerpo de la patente la secuencia de ADN y aminoácidos correspondiente a la EPO inmadura (péptido señal + secuencia de EPO), situación usual en las comunicaciones científicas de los expertos en la materia (Kelly, W et al. Erythropoietin, Biochemistry of. In Encyclopedia of Hormones 2003, pp. 561-569) Por tal motivo, se observa que existe una diferencia de 27 AA entre la numeración de las modificaciones y la numeración de las secuencias originalmente presentadas en la memoria.

En consecuencia, se incorporan las secuencias correspondientes a la proteína madura, lo que consecuentemente se enmiendan las secuencias para atender la observación del Examinador que se contesta. Con lo que queda resuelta la objeción.



4. Examen de Patentabilidad

4.1. Nivel Inventivo

La generación de muteínas mediante la inserción de sitios de N-glicosilación si bien ya fue utilizada en la bibliografía científica, incluso por los mismos inventores de la presente invención, para incrementar la vida media de las proteínas terapéuticas y así su actividad biológica in vivo, dicha modificación **NUNCA** fue empleada para bloquear sitios moleculares responsables de determinadas acciones biológicas a nivel de interacciones con receptores celulares. A partir de D1 (Patente US20070072795) se pone en evidencia la incorporación de modificaciones químicas (residuos de polietilenglicol) y bioquímicas (residuos glicosídicos) para incrementar la vida media de eritropoyetina, pero lo logra en sitios moleculares que no bloquean la actividad eritropoyética sino, contrariamente, la preservan. Nuestra invención se diferencia notablemente ya que los sitios de N-glicosilación fueron incorporados en regiones moleculares que incrementan la vida media, pero bloquean la actividad biológica eritropoyética. Es decir, el efecto técnico es totalmente diferente e inesperado con respecto a lo que muestran los autores de la patente D1. La inhibición completa de la actividad eritropoyética lograda por las muteínas de la presente invención y no enunciadas en la patente D1, contrasta con la preservación de la misma, tal como se evidencia en la patente D1.

La publicación D3 de Elliott et al. estudia la eficiencia de la N-glicosilación en sitios susceptibles para que ella se lleve a cabo. Evalúa el efecto de la posición en la secuencia o estructura primaria de la proteína, la presencia de aminoácidos que circundan el sitio y, en particular enfatiza en la velocidad entre la adquisición de la estructura terciaria y la glicosilación propiamente dicha. Utiliza la EPO para este proceso, pero de ninguna manera modifica los sitios propuestos en la presente invención ni busca el bloqueo de su actividad eritropoyética.



Por los motivos antes expuestos, ni el documento D1 ni D3 afectan la altura inventiva de nuestro descubrimiento, el cual a través de la modificación K45N y N47T ha logrado una independencia absoluta de lo conocido en la literatura y expone su capacidad para la tan ansiada búsqueda de bloquear sitios responsable de la eritropoyesis de modo de disponer de un biofármaco que demuestre actividad en neuroprotección y neuroplasticidad sin los efectos perjudiciales de la actividad biológica eritropoyética, que es la función medular en la biología de eritropoyetina.

Finalmente, la publicación D2 (Gan et al.) expone la modificación S104I que coincide con la modificación propuesta en nuestro pliego reivindicatorio con respecto a la mutación S104N. De ninguna manera se quiso emular la modificación por ellos propuesta, sino que naturalmente se dio en la búsqueda de incorporar sitios de N-glicosilación, en este caso una asparagina en la posición -2 a la Thr106 de modo que quede configurado el sitio de adición de N-glicano (N-X-T, donde N es asparagina, X cualquier aminoácido excepto prolina y T es treonina). Asimismo, el trabajo de Gan et al. cambia un aminoácido hidrofílico (serina) por uno hidrofóbico (Isoleucina) que suele afectar bastante la integridad estructural mientras que en nuestra solicitud mostramos la modificación de serina por otro fisicoquímicamente más relacionado como asparagina que también es hidrofílico lo que permitiría sustentar que conservamos la estructura terciaria (hecho comprobado por ensayo de dicróismo circular y thermal shift). Cabe destacar que, la modificación permitió incorporar una modificación postraducciona (glicano, que fue comprobado por ensayo de digestión parcial con N-glicanasa) que no se encuentra en la EPO natural ni en la modificada por los autores de D2. Dicho glicano aporta mayor hidrofiliidad a la región, lo que la diferenciaría aún más de las propiedades fisicoquímicas generada por la modificación S104I de los autores de D2. Por otro lado, y como es conocido, la adición de glicanos incrementa el tiempo de vida media que se extrapola con condiciones de formulación y dosificación del futuro medicamento que no son aportadas por la modificación realizada por Gan et al. Al



mismo tiempo, hemos observado con la modificación S104N de nuestra molécula un accionar neurobiológico relacionado no sólo con la neuroprotección como demuestran tales autores, sino con la neuroplasticidad tanto en ensayos *in vitro* como *in vivo*, con resultados altamente prometedores para desarrollar un bioterapéutico de interés en aspectos relacionados con enfermedades neurodegenerativas donde la neuroplasticidad se encuentra afectada.

A continuación, se resume un ensayo no mostrado en la patente y que contiene resultados muy interesantes para la respuesta a la vista de patente en un modelo de neuroplasticidad *in vivo* (Ensayo 1_Archivo de evidencia científica):

En ensayos con ratones CF-1 sanos inyectados por vía ip. con la muteína S104N o con rhEPO demostraron un incremento promedio en la extensión total del árbol dendrítico en neuronas hipocampales de 130% o de 54%, respectivamente, con respecto al control inyectado con solución salina de fosfatos. Asimismo, se observó que el total de intersecciones entre dichas neuronas creció un promedio de 63% con la muteína o un 26% con rhEPO. Esto resulta indicativo de la importante acción a nivel de neuroplasticidad de la muteína mencionada, efecto técnico que de NINGUNA manera fue demostrado en el trabajo de Gan et al., e incrementa la relevancia de este potencial medicamento para patologías con neuroplasticidad comprometida.

Ninguno de los tres trabajos (D1, D2 y D3) mencionados por el evaluador permite a un experto medio en la materia llegar a la conclusión de que la mutación de los aminoácidos propuestos en la presente invención genera un sitio que al decorarse con glicanos bloquea la actividad biológica eritropoyética. Aún más, NINGUNO de los documentos señalados menciona dicho sitio, ni que su modificación postraducciona con glicanos bloquea la actividad eritropoyética. Por otro lado, D1 y D2 mencionan actividades neuroprotectoras pero no neuroplásticas que fueron observadas con la muteína K45N/N47T al igual que con



las muteínas S104N y G151N/L153T y cuyos resultados se muestran en la presente respuesta a la vista de la solicitud de patente.

Conclusiones

Con las enmiendas realizadas a las reivindicaciones y los argumentos proporcionados anteriormente, el solicitante considera subsanadas las objeciones del examinador.

En consecuencia y luego de haber presentado los argumentos necesarios y haber realizado las modificaciones pertinentes en el capítulo reivindicatorio de la presente invención, queda evidenciado que la invención cumple con los requisitos de patentabilidad definidos en la Decisión 486 de 2000.

Cordialmente,

Juan Carlos Cuesta Quintero

C.C. 79.339.809 de Bogotá

T.P. 43.273 del C.S. de la J.

Anexos: Comprobante de pago por modificación de la solicitud.
Capítulo reivindicatorio modificado con control de cambios.
Capítulo reivindicatorio modificado en limpio.
Listado de secuencias modificada.

REIVINDICACIONES

1. Una eritropoyetina humana modificada con actividad eritropoyética menor al 0,5 % en relación a una eritropoyetina humana, que mantiene su capacidad neuroprotectora y neuroplástica, caracterizada porque comprende ~~al menos una~~ mutación de un sitio de unión al ~~un~~ receptor homodimérico ~~e heterodimérico~~ mediante la incorporación de sitios consenso de glicosilación, y caracterizada porque dicha mutación es seleccionada del conjunto comprendido por:

a. Lys45Asn y Asn47Thr;

~~b. Tyr15Asn y Leu17Thr;~~

~~c.~~ Ser104Asn;

~~d. Ala98Asn y Ser100Thr;~~

~~e. Thr106Asn y Leu108Thr;~~

~~f. Leu149Thr;~~

~~g.~~ Gly151Asn y Leu153Thr;

~~h. Arg76Asn y Gln78Thr;~~

~~i. Glu72Asn y Val74Thr;~~

~~j. Glu62Asn y Trp64Thr;y~~

~~Gln65Asn y Leu67Thr.~~

y donde dicha eritropoyetina humana comprende una secuencia de aminoácidos seleccionada del conjunto comprendido por SEQ ID N°4, SEQ ID N°18, y SEQ ID N°24.

~~k.~~

~~2. La eritropoyetina humana modificada de la reivindicación 1 donde dichos sitios de unión al receptor son seleccionados del conjunto de aminoácidos comprendidos por las posiciones: 45-47, 15-17, 104, 98-100, 106-108, 149, 151-153, 76-78, 72-74, 62-64, 65-67 y sus combinaciones.~~

~~La eritropoyetina humana modificada de la reivindicación 1 caracterizada porque dicha mutación es seleccionada del conjunto comprendido por:~~

~~a.d. Lys45Asn y Asn47Thr;~~

~~b.e. Tyr15Asn y Leu17Thr;~~

~~c.f. Ser104Asn;~~

~~d.g. Ala98Asn y Ser100Thr;~~

~~e.h. Thr106Asn y Leu108Thr;~~

~~f.i. Leu149Thr;~~

~~g.j. Gly151Asn y Leu153Thr;~~

~~h.k. Arg76Asn y Gln78Thr;~~

~~i.l. Glu72Asn y Val74Thr;~~

~~j.m. Glu62Asn y Trp64Thr;y~~

~~k.n. Gln65Asn y Leu67Thr.~~

~~3. La eritropoyetina humana modificada de la reivindicación 1 caracterizada porque comprende una actividad~~

~~eritropoyética de hasta el 0,2% en relación a una eritropoyetina humana.~~

~~4. La eritropoyetina humana modificada de la reivindicación 1 caracterizada porque comprende nula actividad eritropoyética.~~

5.2. La eritropoyetina humana modificada de la reivindicación 1 caracterizada porque es codificada por una secuencia de ADN que comprende una secuencia de nucleótidos seleccionada del conjunto comprendido por: ~~SEQ ID N°1, SEQ ID N°3, SEQ ID N°7, SEQ ID N°9, SEQ ID N°11, SEQ ID N°13, SEQ ID N°15, SEQ ID N°17, SEQ ID N°19, SEQ ID N°21~~ y SEQ ID N°23.

~~6. La eritropoyetina humana modificada de la reivindicación 1 caracterizada porque comprende una secuencia de aminoácidos seleccionada del conjunto comprendido por: SEQ ID N°2, SEQ ID N°4, SEQ ID N°8, SEQ ID N°10, SEQ ID N°12, SEQ ID N°14, SEQ ID N°16, SEQ ID N°18, SEQ ID N°20, SEQ ID N°22 y SEQ ID N°24.~~

7.3. Un ácido nucleico que codifica para la eritropoyetina humana modificada de la reivindicación 1 caracterizada porque comprende al menos la secuencia de nucleótidos seleccionada del conjunto comprendido por: ~~SEQ ID N°1, SEQ ID N°3, SEQ ID N°7, SEQ ID N°9, SEQ ID N°11, SEQ ID N°13, SEQ ID N°15, SEQ ID N°17, SEQ ID N°19, SEQ ID N°21~~ y SEQ ID N°23.

8.4. Un vector de transformación, caracterizado porque comprende el ácido nucleico de la reivindicación 3 ~~la secuencia de nucleótidos seleccionada del conjunto comprendido por: SEQ ID N°1, SEQ ID N°3, SEQ ID N°7, SEQ~~

~~ID N°9, SEQ ID N°11, SEQ ID N°13, SEQ ID N°15, SEQ ID N°17, SEQ ID N°19, SEQ ID N°21 y SEQ ID N°23.~~

9.5. Un vector de expresión, caracterizado porque comprende el ácido nucleico de la reivindicación 3~~la secuencia de nucleótidos seleccionada del conjunto comprendido por: SEQ ID N°1, SEQ ID N°3, SEQ ID N°7, SEQ ID N°9, SEQ ID N°11, SEQ ID N°13, SEQ ID N°15, SEQ ID N°17, SEQ ID N°19, SEQ ID N°21 y SEQ ID N°23.~~

10.6. Un vector lentiviral, caracterizado porque comprende el ácido nucleico de la reivindicación 3~~la secuencia de nucleótidos seleccionada del conjunto comprendido por: SEQ ID N°1, SEQ ID N°3, SEQ ID N°7, SEQ ID N°9, SEQ ID N°11, SEQ ID N°13, SEQ ID N°15, SEQ ID N°17, SEQ ID N°19, SEQ ID N°21 y SEQ ID N°23.~~

11.7. Una célula genéticamente modificada que comprende el ácido nucleico de la reivindicación 3~~una molécula de ácido nucleico seleccionada del conjunto comprendido por: SEQ ID N°1, SEQ ID N°3, SEQ ID N°7, SEQ ID N°9, SEQ ID N°11, SEQ ID N°13, SEQ ID N°15, SEQ ID N°17, SEQ ID N°19, SEQ ID N°21 y SEQ ID N°23.~~

12.8. La célula genéticamente modificada de la reivindicación 12.6 caracterizada porque comprende una línea celular animal o vegetal o cualquier huésped celular que permita adición de N-glicanos.

13.9. La célula genéticamente modificada de la reivindicación 12.7 caracterizada porque comprende una línea celular animal.

14.10. La célula genéticamente modificada de la reivindicación 12.7 caracterizada porque es seleccionada

del conjunto comprendido por CHO.K1, HEK293, NS0, BHK-21 y HeLa.

~~15.~~11. Una composición farmacéutica caracterizado porque comprende una concentración desde 50 hasta 400 ng/ml ~~cantidad terapéuticamente eficaz~~ de la eritropoyetina humana modificada de la reivindicación 1.

~~16.~~12. Una composición farmacéutica caracterizado porque comprende una concentración desde 50 hasta 400 ng/ml ~~cantidad terapéuticamente eficaz~~ de la eritropoyetina humana modificada de la reivindicación 1 para el tratamiento de patologías seleccionadas del conjunto comprendido por Alzheimer, Parkinson, esclerosis amiotrófica lateral (ELA), enfermedades de las neuronas motoras, enfermedad de Huntington, atrofias espinocerebelosas, enfermedad de Creutzfeld-Jakob, enfermedades incapacitantes como la depresión o la esquizofrenia, enfermedades del desarrollo como el síndrome de Down, daño del tejido nervioso por los accidentes cerebrovasculares (ACV) y las traumatismos cráneo-encefálicos.

~~17. Eritropoyetina humana modificada de la reivindicación 1 que en una concentración desde 50 hasta 400 ng/ml comprende tiene actividad contra patologías seleccionadas del conjunto comprendido por Alzheimer, Parkinson, esclerosis amiotrófica lateral (ELA), enfermedades de las neuronas motoras, enfermedad de Huntington, atrofias espinocerebelosas, la enfermedad de Creutzfeld-Jakob, enfermedades incapacitantes como la depresión o la esquizofrenia, enfermedades del desarrollo como el síndrome de Down, daño del tejido nervioso por~~

~~los accidentes cerebrovasculares (ACV) y las traumatismos
cráneo-encefálicos.~~

~~18.~~13. Un proceso de obtención de la eritropoyetina humana modificada de la reivindicación 1, caracterizado porque comprende los siguientes pasos:

- a. disponer de la ~~secuencia~~ de ácidos nucleicos que codifican dicha eritropoyetina humana modificada y que comprende al menos una secuencia de nucleótidos seleccionada del conjunto comprendido por: SEQ ID N°3, SEQ ID N°17, y SEQ ID N°23.~~—~~;
- b. construir, al menos, un vector de cotransfección de células empaquetadoras;
- c. cotransfectar dichas células empaquetadoras productoras de partículas lentivirales que contienen dicha secuencia de ácido nucleico que codifica para dicha eritropoyetina humana modificada;
- d. cosechar dichas partículas lentivirales producidas por las células empaquetadoras del paso c-;
- e. transducir células capaces de expresar y plegar la eritropoyetina humana modificada con dichas partículas lentivirales de dicho paso d-;
- f. seleccionar las células del paso e- que comprendan la secuencia de ácido nucleico que codifica para dicha eritropoyetina humana modificada;
- g. cultivar las células del paso f- para que expresen dicha eritropoyetina humana modificada y;

h. aislar y purificar dicha eritropoyetina humana modificada.

~~19.~~ El proceso de la reivindicación ~~18~~, donde en dicho paso a-, dichas secuencias de ácido nucleico son seleccionadas del conjunto comprendido por SEQ ID N°1, SEQ ID N°3, SEQ ID N°7, SEQ ID N°9, SEQ ID N°11, SEQ ID N°13, SEQ ID N°15, SEQ ID N°17, SEQ ID N°19, SEQ ID N°21 y SEQ ID N°23.

~~20.~~14. El proceso de la reivindicación ~~18~~11~~13~~, donde dicho paso b- comprende un vector que permite la entrada de la partícula lentiviral en la célula; un vector que codifica para: una proteína de matriz, una cápside, una proteasa, una transcriptasa inversa y una integrasa; un vector de transferencia que contiene la secuencia de eritropoyetina humana modificada; y un vector que induce la exportación nuclear de dicho vector de transferencia.

~~21.~~15. El proceso de la reivindicación ~~18~~11~~13~~, donde en dicho paso e-, dichas células son seleccionadas del conjunto comprendido por CHO.K1, HEK293, NS0, BHK-21 y HeLa.

~~22.~~16. El proceso de la reivindicación ~~18~~11~~13~~, donde dicho paso h-, es una purificación por inmunoafinidad que comprende un anticuerpo anti-rhEPO, y un eluyente.

~~23.~~17. El proceso de la reivindicación ~~22~~14, donde dicho eluyente es seleccionado del conjunto comprendido por glicina, ácido acético - NaCl, sales de acetato, ácido cítrico, sales de fosfato, etanol, isopropanol, dioxano, etilenglicol, tris/HCl, y sus mezclas.

24.18. El proceso de la reivindicación 2214, donde dicho eluyente es seleccionado del conjunto comprendido por glicina, ácido acético- NaCl.

25.19. El proceso de la reivindicación 2214, donde dicho eluyente es seleccionado del conjunto comprendido por glicina 0,1 M pH=2; glicina 0,15 M pH=2,5 y ácido acético 0,2 M, NaCl 0,15 M pH=3.

REIVINDICACIONES

1. Una eritropoyetina humana modificada con actividad eritropoyética menor al 0,5 % en relación con una eritropoyetina humana, que mantiene su capacidad neuroprotectora y neuroplástica, caracterizada porque comprende la mutación de un sitio de unión al receptor homodimérico mediante la incorporación de sitios consenso de glicosilación, y caracterizada porque dicha mutación es seleccionada del conjunto comprendido por:

a. Lys45Asn y Asn47Thr;

b. Ser104Asn;

c. Gly151Asn y Leu153Thr;

y donde dicha eritropoyetina humana comprende una secuencia de aminoácidos seleccionada del conjunto comprendido por SEQ ID N°4, SEQ ID N°18, y SEQ ID N°24.

2. La eritropoyetina humana modificada de la reivindicación 1 caracterizada porque es codificada por una secuencia de ADN que comprende una secuencia de nucleótidos seleccionada del conjunto comprendido por: SEQ ID N°3, SEQ ID N°17, y SEQ ID N°23.

3. Un ácido nucleico que codifica para la eritropoyetina humana modificada de la reivindicación 1 caracterizada porque comprende al menos la secuencia de nucleótidos seleccionada del conjunto comprendido por: SEQ ID N°3, SEQ ID N°17, y SEQ ID N°23.

4. Un vector de transformación, caracterizado porque comprende el ácido nucleico de la reivindicación 3.
5. Un vector de expresión, caracterizado porque comprende el ácido nucleico de la reivindicación 3.
6. Un vector lentiviral, caracterizado porque comprende el ácido nucleico de la reivindicación 3.
7. Una célula genéticamente modificada que comprende el ácido nucleico de la reivindicación 3.
8. La célula genéticamente modificada de la reivindicación 6 caracterizada porque comprende una línea celular animal o vegetal o cualquier huésped celular que permita adición de N-glicanos.
9. La célula genéticamente modificada de la reivindicación 7 caracterizada porque comprende una línea celular animal.
10. La célula genéticamente modificada de la reivindicación 7 caracterizada porque es seleccionada del conjunto comprendido por CHO.K1, HEK293, NS0, BHK-21 y HeLa.
11. Una composición farmacéutica caracterizado porque comprende una concentración desde 50 hasta 400 ng/ml de la eritropoyetina humana modificada de la reivindicación 1.
12. Una composición farmacéutica caracterizado porque comprende una concentración desde 50 hasta 400 ng/ml de la eritropoyetina humana modificada de la reivindicación 1 para el tratamiento de patologías seleccionadas del conjunto comprendido por Alzheimer, Parkinson, esclerosis amiotrófica lateral (ELA), enfermedades de las neuronas

motoras, enfermedad de Huntington, atrofas espinocerebelosas, enfermedad de Creutzfeld-Jakob, enfermedades incapacitantes como la depresi3n o la esquizofrenia, enfermedades del desarrollo como el s3ndrome de Down, da3o del tejido nervioso por los accidentes cerebrovasculares (ACV) y las traumatismos cr3neo-encef3licos.

13. Un proceso de obtenci3n de la eritropoyetina humana modificada de la reivindicaci3n 1, caracterizado porque comprende los siguientes pasos:

- a. disponer de la secuencia de 3cidos nucleicos que codifican dicha eritropoyetina humana modificada y que comprende al menos una secuencia de nucle3tidos seleccionada del conjunto comprendido por: SEQ ID N33, SEQ ID N317, y SEQ ID N323.;
- b. construir, al menos, un vector de cotransfecci3n de c3lulas empaquetadoras;
- c. cotransfectar dichas c3lulas empaquetadoras productoras de part3culas lentivirales que contienen dicha secuencia de 3cido nucleico que codifica para dicha eritropoyetina humana modificada;
- d. cosechar dichas part3culas lentivirales producidas por las c3lulas empaquetadoras del paso c-;
- e. transducir c3lulas capaces de expresar y plegar la eritropoyetina humana modificada con dichas part3culas lentivirales de dicho paso d-;

- f. seleccionar las células del paso e- que comprendan la secuencia de ácido nucleico que codifica para dicha eritropoyetina humana modificada;
 - g. cultivar las células del paso f- para que expresen dicha eritropoyetina humana modificada y;
 - h. aislar y purificar dicha eritropoyetina humana modificada.
14. El proceso de la reivindicación 13, donde dicho paso b- comprende un vector que permite la entrada de la partícula lentiviral en la célula; un vector que codifica para: una proteína de matriz, una cápside, una proteasa, una transcriptasa inversa y una integrasa; un vector de transferencia que contiene la secuencia de eritropoyetina humana modificada; y un vector que induce la exportación nuclear de dicho vector de transferencia.
15. El proceso de la reivindicación 13, donde en dicho paso e-, dichas células son seleccionadas del conjunto comprendido por CHO.K1, HEK293, NS0, BHK-21 y HeLa.
16. El proceso de la reivindicación 13, donde dicho paso h-, es una purificación por inmunoafinidad que comprende un anticuerpo anti-rhEPO, y un eluyente.
17. El proceso de la reivindicación 14, donde dicho eluyente es seleccionado del conjunto comprendido por glicina, ácido acético - NaCl, sales de acetato, ácido cítrico, sales de fosfato, etanol, isopropanol, dioxano, etilenglicol, tris/HCl, y sus mezclas.

18. El proceso de la reivindicación 14, donde dicho eluyente es seleccionado del conjunto comprendido por glicina, ácido acético- NaCl.
19. El proceso de la reivindicación 14, donde dicho eluyente es seleccionado del conjunto comprendido por glicina 0,1 M pH=2; glicina 0,15 M pH=2,5 y ácido acético 0,2 M, NaCl 0,15 M pH=3.

SEQUENCE LISTING

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Consejo Nacional de Investigaciones Científicas y Técnicas
Universidad Nacional de San Martín

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20 25 30

Cys Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe
35 40 45

Tyr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala Val Glu Val Trp
50 55 60

Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu
65 70 75 80

Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp
85 90 95

Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu
100 105 110

Gly Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala
115 120 125

Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val
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Cys Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Asn Val Thr Phe
35 40 45

Tyr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala Val Glu Val Trp

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60

Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu
65 70 75 80

Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp
85 90 95

Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu
100 105 110

Gly Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala
115 120 125

Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val
130 135 140

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Cys Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe
35 40 45

Thr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala Val Glu Val Trp
50 55 60

Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu
65 70 75 80

Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp
85 90 95

Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu
100 105 110

Gly Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala
115 120 125

Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val
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Tyr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala Val Asn Val Thr
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Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu
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Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp
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Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu
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Gly Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala
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Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val
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Gly Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala	115	120	125
Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val	130	135	140
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Tyr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala Val Glu Val Trp
50 55 60

Gln Gly Leu Ala Leu Leu Ser Asn Ala Thr Leu Arg Gly Gln Ala Leu
65 70 75 80

Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp
85 90 95

Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu
100 105 110

Gly Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala
115 120 125

Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val
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Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Asn Gly Thr Ala Leu
65 70 75 80

Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp
85 90 95

Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu
100 105 110

Gly Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala
115 120 125

Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val
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          20           25           30

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Cys Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe
          35           40           45

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Tyr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala Val Glu Val Trp
50           55           60

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Erythropoietin: Structure, Control of Production, and Function

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I. INTRODUCTION

Hemopoiesis counterbalances the continuous loss of aged blood cells. Young progeny are generated by myeloid and lymphoid progenitors that originate from a small pool of pluripotent stem cells. Blood cell counts are fairly constant in health. The rates of the proliferation and differentiation of blood cell lineage-specific progenitors can greatly increase in response to disorders such as hemorrhage or infection.

Hemopoiesis is controlled by several glycoprotein hormones and paracrine peptides. Apart from erythropoietin, these include thrombopoietin (415), at least three different myeloid colony-stimulating factors (GM-CSF, G-CSF, M-CSF) in the granulocytic-monocytic lineage (122, 431, 522), and the various interleukins, which not only activate the lymphocytic system but also modulate the rate of proliferation of myeloid progenitors (21, 431, 489, 522). Several of the human

genes coding for hemopoietic growth factors have been isolated and cloned recently. The recombinant proteins are available for studies of their structure and function and for therapeutic purposes.

Erythropoietin is an essential growth factor in the erythrocytic lineage. One percent of all red blood cells are destroyed daily and replaced by reticulocytes in healthy humans. The basal production rate of $2\text{--}3 \times 10^{11}$ cells/day greatly increases when the blood O_2 availability is lowered. Lack of O_2 induces erythropoietin gene expression in the kidneys (38, 52, 586) and the liver (38, 52). The plasma level of erythropoietin may rise 1,000-fold above normal in severe anemia or hypoxemia.

Purification of a few milligrams of the hormone from human urine was accomplished by Miyake et al. (453) in 1977. This work was a landmark in erythropoietin research. The pure urinary protein enabled investigators to carry out the first reliable radioimmunoassays for the hormone (95, 228, 597) instead of the laborious

and less specific traditional bioassays. The identification of the amino acid sequence of tryptic fragments of the pure urinary protein was an important step toward the cloning of the erythropoietin gene and its *in vitro* expression in mammalian cells (300, 374, 391). Studies utilizing erythropoietin cDNA probes and pure recombinant human erythropoietin have allowed new insights into the chemistry, production, and action of the hormone. In addition, the human and the murine erythropoietin receptor genes have been cloned and structurally characterized (332, 705; for review see Ref. 110).

As was shown in the initial clinical trials (162, 688), replacement therapy with recombinant human erythropoietin is very beneficial in treatment of the anemia of chronic renal failure (6, 9), because it abolishes the need for blood transfusions. In addition, the application of recombinant erythropoietin appears to be useful in other types of anemia, such as in rheumatoid arthritis, acquired immunodeficiency syndrome (AIDS), or malignancies.

This review summarizes current knowledge of the physiology of the production and action of erythropoietin in mammals and points out questions that still need to be answered. Although the whole story comprises three temporal periods, only the third of these is described in detail, beginning with the successful purification of human erythropoietin in 1977 (453). Only brief chronologic information is given on major advances during the first period, in which the idea of the humoral control of erythropoiesis was developed, and the second, beginning in 1950, in which the existence of the erythropoietic hormone was proven. With respect to the early literature, readers are referred to previous reviews (16, 184, 204, 244, 253, 309, 362, 592). In addition, specific information is available on historical aspects (152, 308), control of erythropoiesis in nonmammalian species (308, 362), the pharmacology of the production of erythropoietin (187), the mechanism of action of the hormone on its target cells (359a, 607), and clinical experience with the recombinant product (6, 101, 175, 314, 524, 576).

II. HISTORICAL BACKGROUND

The idea of the existence of a humoral factor controlling erythropoiesis probably originated from two new concepts that were defined by Claude Bernard and co-workers in Paris about 100 years ago. First, it was recognized that the proliferative and metabolic activities of tissues are partly under the control of endocrine glands, and attempts were undertaken to isolate hormones. Second, the consequences of a lack of O₂ in the arterial blood were beginning to be understood. Paul Bert in collaboration with the physician Denis Jourdanet carried out studies at altitude and in decompression chambers that showed that the physiological effects of gasses depend on their partial pressures and that mountain sickness is due to hypoxemia (35; for review see Refs. 152, 342). Bert (35, 36) recognized the elevated O₂ capacity of the blood of altitude residents but considered it to be inherited. The acute effect of hypox-

emia on erythropoiesis was first reported by the French histologist Viault (663) in 1890, who noted that the number of erythrocytes in his blood increased from 5 to 8 × 10⁶/μl within 3 wk during an expedition to the highlands of Morococha (4,400 m) in Peru. In an interesting lecture on the beneficial effects of the treatment of tuberculous and anemic patients in alpine health resorts, Friedrich Miescher (432) in 1893 summarized the early observations of the stimulation of erythropoiesis at altitude. Subsequently, physicians attempted to stimulate erythropoiesis in anemic patients by lowering their alveolar ventilation (364). More recently, physical training of athletes has been practiced at altitude to increase the blood hemoglobin concentration and so improve the endurance performance.

The hypothesis of the control of erythropoiesis by a humoral factor ("hemopoietine") was first formulated by Carnot and Deflandre (72, 73) in 1906. They observed an increase in the number of red blood cells by 20–40% in rabbits 2 days after they received a single injection of a few milliliters of serum from slightly anemic donor animals. This rapid and powerful effect on red blood cell number was not confirmed on careful reinvestigation (244). It is known today that there is a delay of several days until true erythrocytosis occurs after the application of erythropoietin. The effects described by Carnot and Deflandre (72, 73) were perhaps caused by hemoconcentration. Because of the lack of convincing data, the concept of the existence of the hemopoietic hormone was not accepted for almost 50 years (for review see Refs. 152, 244, 308). From this early period, noteworthy reports include those by Sandor (559), who in 1932 demonstrated reticulocytosis ("polychromatophilic macrocytes") in rabbits injected with serum from hypoxic donors, and by Krumdieck (362a), who in 1943 reported an increased number of red blood cell precursors in the bone marrow of the recipients of such sera. In 1948, Bonsdorff and Jalavisto (54) introduced the name "erythropoietin," implying restricted action of the humoral factor on red blood cell production.

In 1950, Reissmann (538) and Ruhenstroth-Bauer (551) showed that erythrocytic hyperplasia of the marrow and reticulocytosis occur in both partners of parabiotic animals when hypoxemia or anemia is induced in one of them. Definite evidence for the existence of erythropoietin was provided by Erslev (150), who demonstrated the occurrence of reticulocytosis and, in the long term, increased hematocrit in rabbits repeatedly infused with large volumes of plasma from severely anemic donor rabbits. The effects observed in this early experimental study are very similar to those seen today in humans treated with pure recombinant erythropoietin. In fact, Erslev (150) in 1953 predicted the potential therapeutic value of the erythropoietic factor once available in purified form. Shortly thereafter, other investigators succeeded in demonstrating erythropoietic activity in plasma (55, 248) and urine (287) from anemic rabbits. Plzak et al. (523) developed an improved bioassay for erythropoietin, which was based on measurements of the incorporation of intravenously administered

radioiron into newly formed red blood cells instead of reticulocyte counts.

Isolated clinical observations of generalized erythrocytic hyperplasia of the marrow in patients suffering from patent ductus arteriosus Botalli suggested a link between hypoxia of the lower part of the body and stimulation of erythropoiesis (577, 616). In 1957, Jacobson et al. (301) reported that the increase in plasma erythropoietin activity does not occur in nephrectomized animals during hypoxia. Lack of erythropoietin was also demonstrated in anemic humans suffering from chronic renal failure (223, 266). Kuratowska et al. (367) and Fisher and Birdwell (189) detected erythropoietin activity in the blood perfusate of isolated kidneys. Nevertheless, for many years the renal synthesis of the hormone was doubted. Instead, hypoxic kidneys were thought to release an enzyme ("erythrogenin") capable of splitting erythropoietin from a hepatic precursor in the blood (245). The erythrogenin hypothesis was disproven with measurements of erythropoietin activity in the serum-free medium of isolated perfused rabbit kidneys (151) and in renal extracts from rats exposed to hypoxia (207, 311) and, unequivocally, with the extraction of erythropoietin mRNA from the kidney (38, 52, 586).

In addition to the kidneys, the liver was established as a site of the production of the hormone (203, 337). However, it was readily clear that the liver is insufficient to substitute for the renal source and, as early as 1974 (167), attempts were undertaken to stimulate erythropoiesis in uremic patients by infusion of erythropoietin-rich human plasma. The well-being of previously anemic patients today is a reward to the investigators (239) who have stayed the long and laborious course from the first purification of animal plasma erythropoietin to the industrial manufacture of recombinant human erythropoietin.

III. BIOCHEMICAL ASPECTS

A. Molecular Biology

The nucleotide sequence of the human erythropoietin gene was reported in 1985 (300, 391). Synthetic oligonucleotide probes were used to screen bacteriophage-borne genomic and human fetal liver cDNA libraries, respectively. In vitro production of biologically active erythropoietin in mammalian cells transfected with the erythropoietin gene linked to an expression vector was demonstrated by Jacobs et al. (300) in stably transformed African green monkey kidney cells (COS-1) and by Lin et al. (391) in Chinese hamster ovary cells (CHO). Erythropoietin gene expression has been accomplished in other mammalian systems, including transformed fetal human kidney (63), human B-lymphoblastoid Namalwa (702), Bowes melanoma cells (341), and baby hamster kidney cells (60, 130, 525, 644). When the human erythropoietin gene is introduced into host cells from nonvertebrates or procaryotes, such as insect cells (526), bacteria (382), or yeast (147), the recombinant products

lack carbohydrate moieties present in the native human hormone. Most studies utilized CHO cultures (63, 113, 534, 560, 561, 632). A CHO cell bank is also the source of the pharmaceutical production of humanlike erythropoietin (63, 143). The CHO cells glycosylate their recombinant product, as does the human organism. In addition, the production of erythropoietin in CHO cell clones can be amplified by genetic engineering, as described elsewhere (29, 143).

The human erythropoietin gene is located on chromosome 7 (525) in the middle of its long arm in the region q11-q22 (380, 675). It exists as a single copy and is composed of five exons and four introns. It encodes a single polypeptide chain consisting of 193 amino acids, including a hydrophobic secretory leader sequence of 27 residues (300, 391). There is no apparent sequence homology with other plasma proteins.

Erythropoietin genes have been isolated from at least two other species. The monkey gene encodes a mature protein of 165 amino acids showing 92% homology with the human sequence (63, 390, 414) but immunologic differences (143). The murine gene is localized on chromosome 5 (373) and encodes a mature protein of 166 amino acids, showing 80% homology with the human sequence (414, 601).

The high degree of homology is not restricted to the amino acid coding region. It is also seen in the flanking 5' region, the first intron, and the 3' flanking region (601). The presence of these conserved sequences has been considered suggestive of their potential *cis*-acting regulatory function in erythropoietin gene expression (39, 292). Imagawa et al. (292) transfected human erythropoietin-producing hepatoma cell cultures (Hep3B) with these sequences inserted 5' and 3' to a reporter gene, coding human growth hormone (GH). Stimulation of the cells by hypoxia or cobaltous ions resulted in increased production of GH. Because similar transfection studies with non-erythropoietin-producing cells (COS 7) were negative for this response, it is thought that the erythropoietin gene is controlled by specific *trans*-acting factors (292). Indeed, from murine kidneys two nuclear factors, a 47-kDa protein and a ribonucleoprotein, could be isolated that bound to the promoter region of the erythropoietin gene. The 47-kDa protein was identified as a constitutive, positive *trans*-acting factor whose action in the uninduced state appeared to be blocked by the ribonucleoprotein. Considered a negative *trans*-acting factor, the ribonucleoprotein was reduced in amount following in vivo induction of the erythropoietin gene by cobalt or hypoxia treatment of the mice (39).

Experimental evidence for *cis*-acting elements in the flanking sequences of the human erythropoietin gene has been provided by studies in transgenic mice. When a cloned DNA fragment that included the complete human erythropoietin gene with 0.4 kb of 5' flanking sequence and 0.7 kb of 3' flanking sequence was microinjected into fertilized mouse eggs, mice with erythrocytosis were generated (589). Human erythropoietin mRNA was detected in the liver of the transgenic mice at the fetal stage and in the liver, kidney, brain, spleen, heart, and lung of adults. Of note, anemia induced in-

creased erythropoietin mRNA levels only in the liver (588, 589). Semenza et al. (588a) have identified a 256-bp 3' flanking sequence that binds hypoxia-inducible nuclear factors. This enhancer element seems to be sufficient for hepatic erythropoietin gene expression, whereas in the kidney other sequences are also required.

B. Structure of Erythropoietin

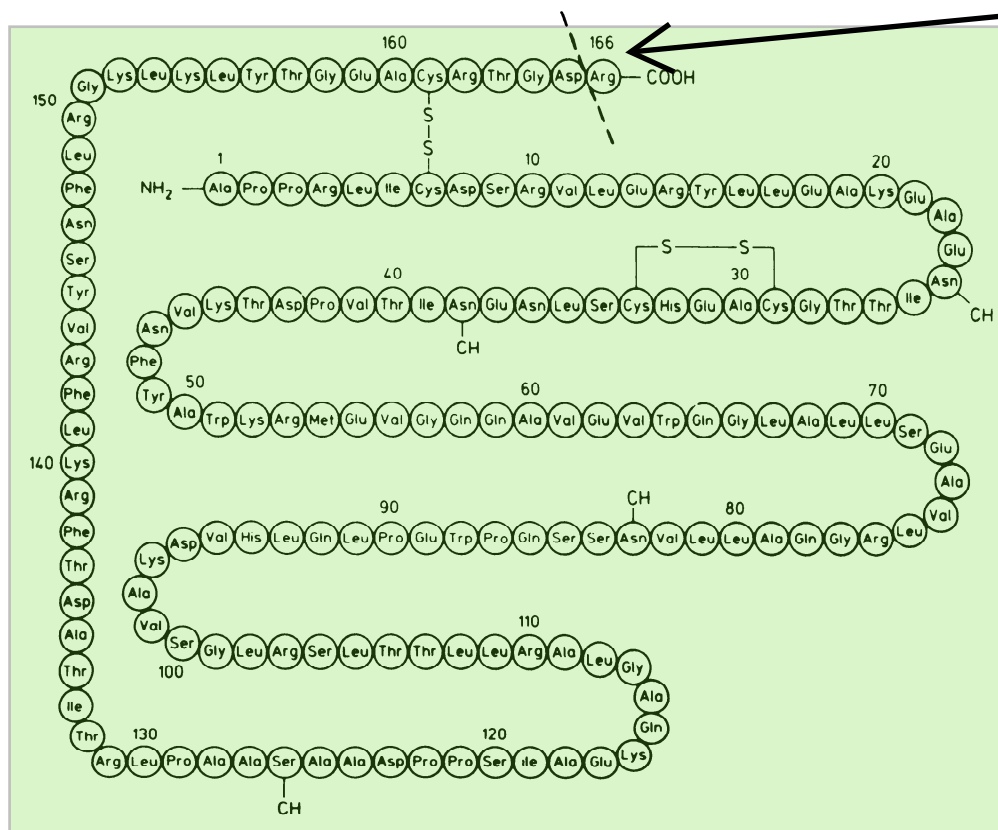
By cotranslational processing, the NH₂-terminal leader sequence of 27 residues is cleaved from the gene product of 193 amino acids (374). In addition, there is posttranslational modification of the molecule (534). Analyses of the COOH terminus by peptide mapping and fast atom bombardment mass spectrometry have shown that the COOH-terminal arginine in position 166 is missing both from human urinary and recombinant erythropoietin (295, 534). Thus the circulating hormone is composed of 165 amino acids (Fig. 1). Two disulfide bridges are formed by the cysteines at the positions 7 and 161 and 29 and 33 (374). Biological activity can be altered by chemical modification of the net charge of distinct amino acid residues (564). It is lost when the sulfhydryl groups are alkylated (672). Studies of the secondary structure of erythropoietin by circular dichroism indicate the presence of 50% α -helices, with the rest being randomly configured (374). It is proposed that two antiparallel pairs of α -helical bundles form a globular structure analogous to GH (31, 49a).

The molecular mass of the peptide backbone of erythropoietin is 18 kDa (374), whereas that of the glycoprotein entity is 30 kDa (113). The carbohydrate portion amounts to 40%. There are three *N*-linked (Asp-24,

-38, and -83) and one *O*-linked (Ser-126) acidic oligosaccharide side chains (60, 145, 374, 560, 632, 645). These consist of fucose, mannose, *N*-acetylglucosamine, galactose, and *N*-acetylneuraminic acid (127). The *N*-linked chains form mainly tetra-antennary complexes (560, 561, 644), whereas the short *O*-linked chain has only four sugar residues (560). There are differences between human individuals in the sugar pattern of the *N*-linked chains (644).

The importance of the carbohydrate portion of erythropoietin is suggested by the conservation of the glycosylation sites in humans, monkeys, and mice. Site-directed mutagenesis of the carbohydrate attachment sites in positions 38, 83, and 126 resulted in lowered secretion of erythropoietin in transfected baby hamster kidney cell cultures (130). However, synthesis of the protein core was impaired possibly due to the amino acid replacement. Erythropoietin secretion is unaltered in mutant CHO cells deficient in UDP-galactose/UDP-*N*-acetylgalactosamine 4-epimerase activity despite the block in *O*-linked glycosylation and in *N*-linked carbohydrate processing (674a).

The resistance of erythropoietin to thermal inactivation depends largely on the completeness of the carbohydrate side chains (645). Earlier studies indicated that enzymatic removal of most of the carbohydrates from human urinary erythropoietin results in aggregation of the protein (127). However, such preparations were still capable of stimulating heme synthesis in bone marrow cultures, indicating that glycosylation is not required for receptor binding and activation of the erythrocytic progenitors (127).



Ver párrafo señalado más arriba.

FIG. 1. Schematic presentation of primary structure of human erythropoietin. Mature hormone is composed of 165 amino acids, having lost carboxy-terminal arginyl residue by posttranslational modification. There are 3 *N*-linked glycosylation sites (CH) at aspartyl residues 24, 38, and 83, and one *O*-linked glycosylation site (CH) at seryl residue 126. [Modified from Lapin and Maxwell (379).]

In vivo, the carbohydrate moiety is important with respect to the metabolic fate of the hormone. Erythropoietin lacking the terminal *N*-acetylneuraminic acid (asialoerythropoietin) is rapidly cleared from the blood by the liver through galactose-binding receptors, whereas it has full or even increased biological activity when assayed in vitro (127, 240, 397, 633, 645). The higher in vitro activity of asialoerythropoietin could be due to its faster binding to the erythropoietin receptor when compared with the intact hormone (294).

Plasma erythropoietin is a relatively heat (532) and pH stable acidic α_2 -globulin (165). There is microheterogeneity in the carbohydrate portion as demonstrated by isoelectric focusing of human plasma erythropoietin, which yields several fractions (166). The isoelectric points of the major components are between pH 3 and 4 (126). The human urinary erythropoietin purified by Miyake et al. (453) consisted of two fractions (α and β) of similar biological activity, molecular mass, and amino acid composition but different electrophoretic mobility and carbohydrate composition (127). Three types of human serum erythropoietin were isolated by gel chromatography, which differed in the ratio of immunoreactivity versus bioactivity and in their molecular masses (594). Separated by zone electrophoresis, 20–30 different forms of immunoreactive erythropoietin have been demonstrated in human serum (684). Of interest are the circadian changes in the pattern of the erythropoietin forms (684). In addition, charge differences were observed when the electrophoretic mobility of erythropoietin from anemic and polycythemic patients was compared (684).

Human urinary and recombinant DNA-derived erythropoietins are identical with regard to amino acid sequence, position of disulfide bonds, glycosylation sites, and secondary structure (113, 145, 295, 534, 560, 644). Although the recombinant products contain no new structural elements, they lack the *N*-acetylneuraminic acid $2\alpha \rightarrow 6$ galactose linkage that is present in the native hormone in addition to the *N*-acetylneuraminic acid $2\alpha \rightarrow 3$ galactose linkage (560, 632). Differences in sialylation may also account for the fact that the specific in vivo biological activity of the purified human urinary erythropoietin amounts to 70,000–80,000 (453, 700) and that of the purified recombinant products amounts to $\sim 200,000$ units/mg peptide. These estimates of specific activity have been based on measurements of protein absorbance at 280 nm, with 1% (wt/wt) solutions giving 8.51 for human urinary erythropoietin (453) and 7.43 for recombinant human erythropoietin (113). The biological activities of the recombinant products can vary depending on the cell type expressing the gene (249). Takeuchi et al. (632) have described a unique CHO cell line, B8–300, that produces two forms of recombinant erythropoietin with different biological activities. Structural analyses of the *N*-linked sugar chains revealed that in addition to the usual product carrying mainly tetra-antennary carbohydrate complexes, erythropoietin with a large portion of biantennary complexes was produced. The higher branching of the *N*-linked su-

gar chains is apparently essential for high in vivo biological activity (632).

C. Assay Methods, Standardization, and Normal Plasma Values

In vivo and in vitro bioassays, radioimmunoassays, and enzyme-linked immunoassays have been applied to measure erythropoietin. The results are expressed in international units (IU). Originally, 1 IU of erythropoietin was defined to elicit the same erythropoiesis-stimulating activity in experimental animals as 5 μ mol cobalt. An international reference preparation (2nd IRP) of impure human urinary erythropoietin (2 IU/mg) was established for in vivo bioassay 20 years ago (20). On behalf of the World Health Organization, a collaborative study was organized recently from the National Institute for Biological Standards and Control, London, aiming to establish a standard for purified DNA-derived human erythropoietin. The specific activity of the new International Standard for Erythropoietin (rDNA-derived) is 130,000 IU/mg fully glycosylated protein.

Before pure erythropoietin became available for immunoassay, in vivo bioassay in polycythemic mice was most commonly used to measure erythropoietin in biological fluids (97, 114, 339, 678). Briefly described, erythrocytosis is first induced in the test mice by 2–3 wk exposure to hypobaric hypoxia or by red blood cell hypertransfusion. Endogenous erythropoiesis is suppressed for several days thereafter. Erythropoietin given in the test samples evokes a proportional increase in red blood cell production. Radioiron is then administered, which is incorporated into newly synthesized hemoglobin. The stimulation of erythropoiesis is quantified by measurement of the radioactivity of red blood cells. The method has been used for assay of erythropoietin in samples from various mammalian sources, although species-dependent differences in the response may exist (44, 480). The in vivo bioassay is relatively specific for erythropoietin. However, it requires many test mice, it is time consuming and expensive, and there is considerable intra- and interassay variance of the results. The detection limit has been reported in the range of 50–25 U/l (91, 178, 311, 339), which is not sensitive enough for the measurement of serum erythropoietin in normal subjects. In two studies applying assay modifications, mean activities in normal human serum amounted to 3 U/l (667) and 8 U/l (156). There is a fairly good correlation between the results obtained by in vivo bioassay and by radioimmunoassay (91, 321, 535, 597).

Various cell culture techniques have been described for bioassay of erythropoietin (cf. Refs. 133, 309, 379). The measurement parameters were usually of proliferative activity or heme synthesis in cultures of mouse, rat, or rabbit bone marrow (182, 237, 360, 363, 550), spleen (378), or fetal liver (58, 115, 132, 475, 528). Serum-free culture systems have been also described (297, 550). In vitro bioassays are rapid, economic, and sensitive enough to demonstrate erythropoietic activity in normal serum. However, their specificity is poor, because

biological fluids often contain toxic substances or factors other than erythropoietin that influence the growth of erythrocytic progenitors (58, 268, 528). With few exceptions (182, 385, 550), estimates of the erythropoietin concentration in serum samples from normal individuals were much higher by *in vitro* bioassay (115, 363, 378, 475, 528) than by *in vivo* bioassay or radioimmunoassay. Cell culture assays may still be of some value in fractionation procedures and for detection of erythropoietin fragments that lack immunologic and biological *in vivo* activity.

Immunologic techniques that are more precise, sensitive, and rapid are today preferred for the measurement of erythropoietin. Reliable immunoassays require either the use of pure erythropoietin as the labeled antigen or of monospecific antibody. Initially, purified human urinary erythropoietin was iodinated for radioimmunoassay and used together with polyvalent antiserum from rabbits immunized with impure human urinary erythropoietin (95, 228, 535, 597, 709). Subsequent studies showed that the native hormone can be replaced by recombinant erythropoietin both as the labeled antigen and for immunization (144, 401). This procedure has been widely adopted (140, 321, 378, 404). In addition, monoclonal antibodies have been raised toward synthetic erythropoietin fragments (619, 627) and the native hormone (454, 680, 690, 701) and been used to develop enzyme-linked immunosorbent assays for erythropoietin (249, 691). Few cases have been reported of invalid estimates due to cross-reactant factors in human serum (102). Of note is the species specificity in the antigen-antibody reaction (228, 693). There is cross reaction but nonparallelism of the assay response dilution lines if erythropoietin samples from nonhuman sources are included in the human test system. Therefore valid immunoassays must use erythropoietin standard from the respective species. This standard should be calibrated by *in vivo* bioassay.

Published values of the mean serum concentration of immunoreactive erythropoietin in nonanemic humans range from 6 to 32 U/l (95, 96, 144, 227, 321, 349, 378, 401, 404, 440, 535, 685, 691, 709). The varied estimates may have resulted from between-laboratory differences in both erythropoietin standard preparations and assay regimes. The serum erythropoietin concentration is similar in healthy women and men (95, 320, 401, 440, 461, 535) despite the difference in the blood hemoglobin concentration. In addition, erythropoietin levels are independent of age (95, 283, 440, 461). With low values before noon and the highest values early in the night, a moderate circadian rhythm has been assessed in normoxic persons (98, 685) and in patients with respiratory failure (442). There is no significant fluctuation in the erythropoietin level during the menstrual cycle (96, 99). Accompanying the development of dilution anemia, the concentration of erythropoietin in serum rises in pregnant women after the third month of gestation, reaching values of ~35–80 U/l (33, 96, 99, 686).

IV. SITES OF PRODUCTION

A. Kidney

Soon after birth the kidneys take over from the liver in becoming the main site of production of circulating erythropoietin. As first shown by Jacobson et al. (301), bilateral nephrectomy inhibits the increase in plasma erythropoietin following hemorrhage or cobalt treatment in adult mammals. The rise in plasma erythropoietin in hypoxia results from *de novo* mRNA and protein synthesis. It is blocked by actinomycin D, puromycin, or cycloheximide (581). Little erythropoietin can be extracted from the renal tissue of normal animals (75, 90, 307, 311, 596). Northern blot analyses proved to be too insensitive to detect erythropoietin mRNA in the kidneys of normoxic mice (52, 372) and rats (38, 585, 586). By RNase protection assay (533, 635a) and competitive polymerase chain reaction (J. Fandrey, personal communication), low levels of erythropoietin mRNA were measured in unstimulated rat kidneys (533, 635a).

Significant amounts of erythropoietin can be extracted from the kidneys of rodents some hours after the induction of hypoxemia (75, 89, 206, 207, 307, 311), anemia (76), carboxyhemoglobinemia (575), or the administration of cobalt (335). In rats exposed to acute hypobaric hypoxia, the renal erythropoietin content rose for ~6 h, then declined despite continued hypoxic stress, and eventually remained moderately elevated above normal (75, 307). Renal erythropoietin mRNA in mice increased within 1.5 h and was maximally expressed by 4–8 h after anemia was induced (52). The maximum level correlated with the severity of the anemia, indicating that the rate of the production of erythropoietin depends on the accumulation of its mRNA. Renal erythropoietin mRNA levels in rats have been also shown to increase after cobalt treatment, bleeding, or exposure to hypobaric hypoxia (38, 586, 635a). Because the level of mRNA depends both on the rate of its synthesis and its degradation, it was important to demonstrate active erythropoietin gene transcription in isolated renal cell nuclei prepared from rats after *in vivo* exposure to hypoxia and cobalt treatment (585).

Several studies have addressed the question as to which renal cells synthesize the hormone. Erythropoietin activity exists mainly in the cortex of hypoxic kidneys (206, 311). Earlier immunofluorescence histochemical studies revealed staining for erythropoietin exclusively in the renal corpuscles (70, 195, 202, 263). Enzyme-immunohistochemical and electron-microscopic studies showed the epithelium of the renal corpuscles to be the only binding site for erythropoietin antibody in human cadaveric kidney (462). In studies with highly specific antiserum against pure human erythropoietin, fluorescence was seen in the renal mesangium of hypoxic rats, while other parts of the kidney and the mesangium of normal rat kidney were negative (218). Rat kidney mesangial cells in culture produce an erythropoietic activity (219, 370), but its identity with erythropoietin

has remained unproven. The demonstration of immunofluorescence in the renal corpuscles of rats some weeks after birth coincided with the switch from the liver to the kidney as the primary site of the production of erythropoietin (263). However, more recent immunohistochemical studies are in contrast with this localization. With purified antiserum to recombinant human erythropoietin, immunofluorescence was observed in tubular cells of hypoxic mouse kidneys (406). In the only investigation with monoclonal antibody raised against human erythropoietin, immunostaining was seen in the endothelium of peritubular blood vessels in the kidney cortex of anemic mice. Neither the renal corpuscles nor the tubules revealed immunoreactivity (624).

According to Northern blot analyses of hypoxic rat kidney extracts, erythropoietin mRNA is clearly expressed outside of the renal corpuscles (586). Both ³⁵S-labeled RNA (357) and DNA (372) probes corresponding to the murine erythropoietin gene code were found to hybridize to peritubular cells in the cortex and the outer medulla of kidneys from anemic mice (357, 372). The number of cells expressing erythropoietin mRNA increased with decreasing hematocrit (359). Hence it has been proposed that when anemia becomes more severe, additional cells are recruited that synthesize erythropoietin, with the individual cell responding maximally once activated (359).

Some of the radiolabeled cells in these studies had an endothelial-like morphology (357), and they contained factor VIII-related antigen (372). Therefore peritubular capillary endothelial cells have been considered the most likely candidates for expressing erythropoietin mRNA in the hypoxic murine kidney. Studies in rats exposed to carbon monoxide have confirmed this localization (368). Nevertheless, the evidence for the peritubular production site is not accepted by all authors (cf. Ref. 544a). Using a ³²P-labeled DNA probe corresponding to part of exon IV of the murine erythropoietin gene, Maxwell et al. (406) recently reported their localization of erythropoietin mRNA in tubular cells of the hypoxic mouse kidney. Although there is no apparent explanation for this discrepancy, it clearly demonstrates that *in situ* hybridization findings require cautious interpretation.

In summary, conflicting opinions still exist with respect to the site of the synthesis of erythropoietin in the kidney. Neither immunohistochemical nor *in situ* hybridization studies have provided a clear answer to this important question. Most authors favor a peritubular origin from cells in the endothelium, but evidence for a tubular origin is also available.

B. Extrarenal Sites

Erythropoiesis is maintained at a low rate in the renoprival state (450, 471). Studies in nephrectomized animals (157, 303, 317) and humans (74, 450, 470, 471) have shown that some erythropoietin is produced extrarenally in response to hypoxia. Similar to renal produc-

tion, the extrarenal formation of erythropoietin increases with the severity of the hypoxic stimulation (15, 157, 211). Structural or functional differences between renal and extrarenal erythropoietin have not been reported.

Aside from kidneys and liver, several other tissues were earlier thought to produce erythropoietin (cf. Ref. 309). Of these, only macrophages, especially in the bone marrow and spleen, have remained a candidate in debate (544a). The evidence for other erythropoietin-producing tissues is not convincing. For example, earlier reports of erythropoietin synthesis in the carotid bodies could not be reproduced (279). The removal of the carotid bodies enhances the plasma erythropoietin level in hypoxemic animals as a consequence of the suppressed respiratory drive and thus lowered arterial O₂ tension (43, 505). The significance of the immunohistochemical demonstration of erythropoietin in the submandibular gland of rodents (176) is uncertain in view of the conflicting findings with this technique in the renal localization. According to recent findings (634), proteolytic degradation of radiolabeled antigen may have feigned the reported erythropoietin immunoreactivity in extracts from salivary glands (89). Attempts have failed to detect erythropoietin mRNA in rat salivary glands (38).

1. Liver

The liver is the primary site of the fetal synthesis of erythropoietin (90, 394, 513, 707). When fetal or newborn lamb livers were transplanted into adult sheep, the transplanted fetal liver produced significantly more erythropoietin than the newborn organ (197). This finding indicates that either the erythropoietin-producing capacity of the liver or its sensitivity to hypoxia decreases after birth. In addition, the perinatal changes in the O₂ affinity of the blood could play a role. The plasma level of erythropoietin in newborn rats increases much less in response to hypoxemia than to anemia, which has been related to the high O₂ affinity of the blood in the neonate (76).

The liver has been known as the main extrarenal site of the production of blood-borne erythropoietin in adults, since hepatectomy was reported to abolish the hypoxia-induced increase in plasma erythropoietin in anephric rats (203, 337). Extrarenal production of erythropoietin in hypoxia is enhanced during recovery from hepatic injury, such as following partial hepatectomy, CCl₄ intoxication, and bile duct ligation (208, 476). It has been proposed that the regenerating liver releases an activity, the so-called hepatic erythropoietic factor, that stimulates liver cells to produce erythropoietin (128).

Erythropoietin mRNA has been demonstrated by Northern transfer analysis of liver extracts from anemic mice. This mRNA amounted to ~17% of that in the kidneys (52). By RNase protection assay, erythropoietin mRNA was detectable in liver from normal rats. Hepatic erythropoietin mRNA accounted for 27–51% of the total in hypoxic rats, with the kidneys accounting for

most of the remainder (635a). Treatment with cobaltous chloride also induces erythropoietin mRNA formation in the liver of rats (38). Evidence for the hepatic synthesis of erythropoietin was provided earlier with the measurement of hormone activity in the perfusate of isolated livers (67, 78, 539). Erythropoietin bioactivity was not clearly detectable in extracts of livers from hypoxic rats (78, 206, 311). Apparently the hormone is not stored in measurable amounts in liver.

The degree of the hepatic production of erythropoietin is species dependent. It can be very low, such as in dogs (cf. Ref. 185). In adult rats rendered anemic (89, 157) or hypoxemic (203, 317) immediately after nephrectomy, the plasma erythropoietin level reached 10–30% of that in comparably hypoxic rats with intact kidneys. However, a much smaller increase in plasma erythropoietin was observed when the hypoxic stimulus was initiated 1 day after nephrectomy (580). The hepatic potential for erythropoietin mRNA synthesis was not impaired in rats 4 wk after subtotal nephrectomy (635a).

Here of interest are the relatively low blood levels of erythropoietin in uremic patients despite severe anemia (74, 83, 84, 421, 470, 508, 597, 709). The question has to be raised as to why the liver is incapable of substituting for the kidneys more effectively. It might be worthwhile studying whether humoral mediators produced in chronic renal diseases inhibit the synthesis of erythropoietin at extrarenal sites.

Fried et al. (208) have considered the bile duct epithelium to be the site of the synthesis of erythropoietin in the liver, because elevated plasma levels of the hormone were measured in rats with bile duct hyperplasia. Other investigators have reported a correlation between erythropoietin activity and hyperplasia of the reticuloendothelial system in anephric rats treated with colloidal carbon or zymosan (513). In immunofluorescence studies, erythropoietin was localized in the Kupffer cells of the extirpated fetal mouse liver and in their tissue culture outgrowths (262, 263). Both immunoreactive and bioactive erythropoietin were detected in Kupffer cell cultures from adolescent rat livers (503). According to a recent *in situ* hybridization study, erythropoietin mRNA expression occurs predominantly in centrilobular epithelial cells in the livers of transgenic and normal mice following the induction of anemia (358).

2. Tissue macrophages

Erythropoiesis proceeds in normoxia, although there is little erythropoietin gene expression in the kidneys and the liver. Because some erythropoietin is always present in the blood, even in patients with polycythemia vera, it would appear that the renal and hepatic production of the hormone is maintained at a low rate in the normoxic state. Some investigators have disputed this and suggested erythropoietin derives from a subpopulation of bone marrow macrophages at the site of erythropoiesis under steady-state conditions (544a). Murine myeloid and splenic macrophages in culture have been reported to produce an erythropoietic activity (544,

545). In normal mouse marrow smears, 30% of all macrophages were found to exhibit erythropoietin gene expression as judged from hybridization studies utilizing biotinylated erythropoietin DNA coupled with streptavidin-gold signal detection (665). On the basis of these findings, it has been argued that the paracrine release of erythropoietin from macrophages maintains erythropoiesis under steady-state conditions, whereas the endocrine production of the hormone in the kidneys and liver is of importance during hypoxic stress (665).

Although attractive, the experimental evidence for the hypothesis that macrophages produce erythropoietin is still incomplete. A quantitative assay of erythropoietin mRNA in bone marrow macrophages has not been published. The cellular origin of erythropoietin mRNA recently detected in rat spleen and lung by RNase protection assay (635a) and by competitive polymerase chain reaction (J. Fandrey, personal communication) still needs to be identified.

V. TISSUE HYPOXIA AS STIMULUS OF ERYTHROPOIETIN PRODUCTION

In addition to maintaining erythropoiesis under steady-state conditions, erythropoietin is important in accelerating the recovery of red blood cell mass after hemorrhage. Erythropoietin levels may rise to 10,000 U/l plasma in severe anemia compared with the normal value of 5–20 U/l. The concentration of red blood cells is not a directly controlled variable in the feedback regulation of erythropoiesis. Instead, the blood O₂ availability is sensed. Tissue hypoxia is the primary stimulus of the production of erythropoietin. The cells controlling the synthesis of the hormone appear to respond to changes in the O₂ capacity, the O₂ tension, and the O₂ affinity of the blood. In addition, the rates of the local blood flow and of the cellular O₂ consumption may play some role.

A. Whole Organism

1. Lowered blood oxygen capacity

Plasma erythropoietin activity increases exponentially with lowered O₂ carrying capacity of the blood. Multiple studies in chronically anemic humans have shown that there is generally an inverse log/linear relationship between serum erythropoietin activity and hemoglobin concentration (24, 95, 96, 116, 286, 321, 439, 475, 608, 631, 685) or hematocrit (14, 158, 159, 422). Quantitative differences exist depending on disease. Patients with hypoplastic anemias have elevated erythropoietin levels for the degree of their anemia (see sect. VII B). On the other hand, patients with chronic inflammatory or malignant diseases tend to have inappropriately low levels of erythropoietin (see sect. IX B).

Acute hemorrhage leads to increased production of erythropoietin in both humans (4, 96, 441) and animals (38, 52, 150, 157, 443, 447, 451, 475). In two independent

studies, the serum concentration of immunoreactive erythropoietin was found to increase by 5 U/l (441) and by 22 U/l (96) in normal humans 24 h after the acute loss of 0.5 l blood, such as occurs in blood donation. The relationship between plasma erythropoietin level and severity of hemorrhage has been studied in detail in rats (443). Because erythropoietin levels started to increase a few hours after a blood loss, hypovolemic circulatory hypoxia has been considered the primary stimulus for the synthesis of the hormone after bleeding (447). Effects of acute isovolemic anemia were studied in rats following exchange transfusion with plasma (318, 478). Compared with bleeding, phenylhydrazine-induced hemolytic anemia of a similar degree caused a more sustained increase in serum erythropoietin in rabbits (426) and rats (157). The important role of the O_2 carrying capacity of the blood in the control of erythropoiesis is further indicated by the reticulocytosis occurring in hypervolemic mice with lowered hematocrit due to overhydration (451).

Conversely, erythropoiesis is suppressed in pseudopolycythemia due to dehydration, as in true polycythemia following the transfusion of blood (451). Plasma erythropoietin levels are lower than normal in blood-transfused normoxic animals (457) and in patients with polycythemia vera (4, 100, 117, 156, 227, 709). The erythropoietin response to hypoxemia is blunted in animals with hypertransfusion polycythemia (15, 302, 347, 478). It remains to be clarified as to why this inhibition is much less pronounced when erythrocytosis is induced endogenously by previous exposure to hypoxia (15).

As noted, the production of erythropoietin is not stimulated in normoxic subjects when hematocrit is extremely elevated. This fact indicates that the O_2 supply at the sensing site is not limited by sluggish blood flow associated with high viscosity (153). In addition, the reduced erythropoietin level in polycythemia vera patients implies that there is no lower threshold in the control of the production of the hormone.

2. Lowered arterial oxygen tension

On lowering the inspiratory O_2 tension, plasma erythropoietin increases exponentially with the degree of hypoxemia, as demonstrated in humans (1, 136, 435) and in experimental animals (1, 318, 478, 581). Both sojourners and natives living at altitude exhibit elevated red blood cell counts (383). Associated with high hematocrit, blood viscosity and cardiac work load increase, eventually leading to a lowered O_2 supply to the tissues (153, 640).

On the other hand, a fall in serum erythropoietin has been noted in anemic patients inspiring almost pure O_2 (96, 148) and in anemic rats exposed to hyperbaric O_2 (478). These observations are of interest in view of the shape of the hemoglobin O_2 binding curve, because the O_2 content of fully oxygenated arterial blood increases very little when the O_2 tension is enhanced above normal.

3. Increased blood oxygen affinity

Evidence for the influence of the O_2 affinity of the blood on erythropoiesis is given with the erythrocytosis in humans with high- O_2 affinity stable mutant hemoglobins (7, 280). The presumed cause of the erythrocytosis is stimulation of the production of erythropoietin, because O_2 dissociates from high- O_2 affinity hemoglobin only at comparably low tissue O_2 tensions. Indeed, the plasma erythropoietin level was shown to rise acutely in rats following exchange transfusion of normal blood with high- O_2 affinity blood (381, 443). Of clinical relevance is the low 2,3-bisphosphoglycerate level, and thus high O_2 affinity, of blood stored for transfusion in acid citrate dextrose. It has been reported that the transfusion of fresh blood causes a greater fall in the plasma erythropoietin activity in anemic patients than the transfusion of stored blood (474). On the other hand, a lowering of the O_2 affinity of the blood is associated with decreased erythropoietin levels, such as in patients with sickle cell anemia (598).

Acute exposure to carbon monoxide is perhaps the most severe stimulus to the *in vivo* production of erythropoietin (318, 574). Compared with phenylhydrazine-induced hemolytic anemia, carboxyhemoglobinemia (50% COHb) caused a 20-fold greater rise of the plasma erythropoietin level in rats at a similarly reduced O_2 capacity of the blood (318). The strong effect of carbon monoxide is probably due to the very high O_2 affinity of partly CO-loaded hemoglobin (318). The increase in blood hemoglobin concentration and red blood cell mass in smoking humans (553) also results from CO binding to hemoglobin. Of note, the inhibition of the *in vitro* production of erythropoietin by carbon monoxide at higher concentrations (234) involves a different mechanism (see sect. VB).

The strong influence of pH on the plasma level of erythropoietin has been related to changes in the O_2 affinity of the blood, too (446). Due to the Bohr effect, a decrease in pH favors the dissociation of O_2 from hemoglobin. The erythropoietin response to anemia (444), hypoxemia (92, 139, 444, 695, 710), carboxyhemoglobinemia (139), and cobalt treatment (445) is lowered in acidotic and enhanced in alkalotic humans and animals. Extrarenal erythropoietin production is also suppressed in acidosis, as demonstrated in anephric rats exposed to hypoxia combined with hypercarbia (710). The reduced formation of erythropoietin in acidotic animals exposed to hypoxic hypoxia has been explained by the increased respiratory rate, resulting in higher arterial O_2 tension (92, 695). Studies in mice exposed to hypobaric hypoxia for 15 min and subsequent normoxia for 2 h have shown that the application of acidic ammonium chloride prevents the increase in plasma erythropoietin only when administered before the hypoxic period. Hence it has been proposed that, rather than the synthesis of erythropoietin, the O_2 sensing process is impaired in acidosis (139). There is also evidence for a direct influence of pH on either the cells of origin of erythropoietin or, if separate, the cells of O_2 sensing. The plasma erythropoietin activity in anemic rats was significantly lower on 24 h

exposure to hypercapnic hypoxia compared with normocapnic hypoxia, while there were only minor differences in arterial O_2 tension and O_2 affinity (444).

At altitude, an increased O_2 affinity of the blood facilitates O_2 loading in the pulmonary circulation. Compared with normal, plasma erythropoietin activity was reduced at 9,930 or 6,050 m simulated altitude in mice previously given cyanate in the drinking water to produce carbamylated, high- O_2 affinity hemoglobin (600). To exclude the possibility of unspecific inhibition of the synthesis of protein by cyanate, studies were carried out in rats after exchange/transfusion with normal or cyanated blood (381). The erythropoietin response in the high- O_2 affinity group was enhanced at sea level and at moderate altitude (4,750 m) but was similar to the response in normal rats at high altitude (7,000 m). Apparently the delivery of O_2 from the high- O_2 affinity blood to the cells controlling the production of erythropoietin was reduced at mild hypoxic hypoxia, but this disadvantage was equalized by an improved oxygenation in the lung at extreme altitude (381).

4. Lowered renal blood flow

Most authors suppose that the plasma level of erythropoietin is governed by the ratio of the renal O_2 supply to demand (30, 155, 204), because the kidneys are the main site of the synthesis of the hormone. In addition, the kidneys would seem to be apt at sensing the O_2 content of the blood. Their O_2 supply is little influenced by changes in cardiac output and arterial blood pressure because of the autoregulation of the renal blood flow. Furthermore, a lowering of the arterial O_2 content or unloading ability is not masked by an increase in the renal blood flow rate (170, 699). Oxygen consumption by the kidneys depends mainly on the rates of renal blood flow, glomerular filtration, and tubular sodium reabsorption. Within physiological limits, a decrease in renal blood flow elicits a proportional reduction in renal O_2 demand. Erslev et al. (155) have pointed out that the unique relationship between renal blood flow and O_2 consumption prevents the development of a vicious cycle in polycythemia, with hyperviscosity of the blood causing lowered perfusion rate, tissue hypoxia, and further stimulation of erythropoiesis.

Increased plasma erythropoietin levels were measured in animals with diminished renal blood flow induced either surgically by renal artery constriction (194, 260, 465, 498, 499) or pharmacologically by the application of vasoconstrictive agents such as angiotensin II (193, 251), norepinephrine, 5-hydroxytryptamine (193, 393), or bombesin (429). The shunt perfusion of venous blood from the right atrium into the renal artery of dogs elicited erythropoietin production, at least in 50% of the animals (509). As detailed in section VB, isolated kidneys generate erythropoietin when perfused with medium at low O_2 tension (151, 191, 501, 504, 533, 578a, 706).

The influence of changes in renal O_2 consumption on the production of erythropoietin is less obvious. It has been proposed that tubulotoxic compounds such as

uranyl nitrate (231), gentamicin (317), amphotericin B (389), organic mercurials (190), and the carbonic anhydrase-inhibiting drug acetazolamide (138, 190) may lower the hypoxia- or cobalt-stimulated increase in plasma erythropoietin by way of decreased renal O_2 utilization. However, this explanation is not fully convincing, because the production of erythropoietin was found unaltered in anemic (540) and hypoxemic (317, 518) rats when glomerular filtration and thus tubular work load was greatly abolished by ureteral ligation.

Whether O_2 sensing and erythropoietin synthesis are accomplished by the same cell is a difficult point, as long as the intrarenal site of the production of the hormone is not clearly known. In addition, the question still needs to be addressed as to whether the renal O_2 sensing system plays a primary or a supportive role in the regulation of erythropoiesis. The plasma erythropoietin level is seldom elevated in humans with renal artery stenosis (cf. Ref. 264). The prevalence of erythrocytosis is only 10%, and the increment in hematocrit is small in the afflicted persons (264). A quantitative analysis of the findings in experimental animals reveals that the stimulation of the production of erythropoietin was in general only moderate (194, 260, 465, 498) or even missing (93) when the O_2 supply to the kidneys alone was lowered by the application of Goldblatt clips. A flow reduction of at least 50% below normal appears to be necessary to elicit some erythropoietin production (194, 498). Pagel et al. (499) measured the O_2 tension of renal mixed venous blood of rats following renal artery constriction. The O_2 tension clearly fell when renal blood flow was reduced below 60% of normal. The O_2 tension in the renal vein was <10 mmHg, with a flow reduction to 10%. Nevertheless, plasma erythropoietin activity increased only from 20 to 69 U/l in 18 h (499). By comparison, an 80-fold greater rise in the plasma erythropoietin level occurred in anemic rats when the blood O_2 capacity was lowered to 25% of normal (498). In addition, rats with reduced renal blood flow produced erythropoietin in high amounts on exposure to hypobaric hypoxia (499).

B. Isolated Organs and Cell Cultures

1. Isolated perfused kidneys and livers

The potential of isolated organs to produce erythropoietin was demonstrated several years ago. Erythropoiesis-stimulating activity was generated during hypoxemic perfusion of kidneys from rabbits (361, 539) or dogs (191, 706) and of livers from rats (67) or rabbits (539). However, the resulting activities were low and hardly detectable by *in vivo* bioassay for erythropoietin. Therefore subsequent perfusion experiments were carried out using so-called "programmed" organs that were obtained from hypoxemic animals (78, 151, 313, 399, 506, 529). Initiated *in vivo*, the *in vitro* formation of erythropoietin by programmed kidneys and livers is enhanced. Programmed rabbit kidneys (151) and programmed rat

livers (78) were also shown to produce erythropoietin during perfusion with serum-free medium.

Measurements of immunoreactive erythropoietin (501, 504, 578a) and erythropoietin mRNA (533) have recently enabled investigators to quantify erythropoietin synthesis in serum-free perfused kidneys from normal rats. The level of immunoreactive erythropoietin in the perfusate was shown to increase markedly within 3 h when the O_2 tension of the recirculating medium was lowered (501, 578a). On the other hand, the degree of the production of erythropoietin was almost independent of the O_2 carrying capacity, with hematocrit ranging from 0.0 to 0.4 (501). Thus the synthesis of erythropoietin appears to be governed primarily by the arterial O_2 tension in the isolated perfused rat kidney. According to measurements of immunoreactive erythropoietin in the perfusion medium (501) and of erythropoietin mRNA in the tissue of isolated perfused rat kidneys (533), little erythropoietin was produced in kidneys during "anemic" perfusion at high flow rate and high O_2 tension. An increase in renal erythropoietin mRNA occurred at lowered perfusion flow rate and normally high arterial O_2 tension, with venous O_2 saturation decreasing from 93 to 87% (533). Of note, a high venous O_2 tension and O_2 saturation may indicate adequacy of the O_2 supply to the kidney as a whole, but not to the individual cells, mainly because of arteriovenous shunt diffusion of O_2 , inhomogeneous blood distribution, and plasma skimming resulting in low hematocrit in medullary vessels. In the study of Ratcliffe et al. (533), the accumulation of erythropoietin mRNA at low flow rate was prevented when the perfusate was gassed with pure O_2 , with venous O_2 saturation increasing to 99%. Taken together, these findings are in keeping with a renal O_2 sensor controlling the synthesis of erythropoietin.

2. Cell cultures

Cell cultures enable one to study biological processes in homogenous tissue independently of humoral and neural signals. Therefore efforts have been made for over 20 years to develop culture systems that are suitable for study of the cellular mechanisms of the production of erythropoietin. A number of renal and nonrenal cell lines and primary cultures have been reported to produce erythropoietin or erythropoietin-like activity (cf. Ref. 316). Among these were cultures of human renal tumors (269, 271, 336, 473, 491, 554, 593, 595, 599, 602, 625, 628, 652, 653), rat kidneys (219, 370), rabbit kidneys (490, 497), bovine kidneys (416, 490), goat kidneys (68), human hepatic tumors (491), rat liver Kupffer cells (503), fetal mouse livers (262, 369, 490, 711, 712), mouse bone marrow macrophages (544, 545, 665), murine erythroleukemia cells (37, 86, 278, 635), and human testicular germ cells (22).

Some of the cultures responded with increased formation of erythropoiesis-stimulating activity when maintained at low O_2 tension in the incubator, including bovine kidney organ cultures (416), rat kidney mesangial cells (370), the human renal carcinoma cell line RC-

1 (599), fetal mouse liver cultures (369), and mouse marrow macrophages (544). Nevertheless, these cell culture systems have proved less appropriate for study of the mechanism by which hypoxia triggers the synthesis of erythropoietin. In the case of organ outgrowths, the erythropoietin-producing cell type was not clearly identifiable (68, 369, 416, 490, 595). In addition, the erythropoietic activity in the culture media was generally very low and, in some studies, could be demonstrated only by *in vitro* bioassay (219, 370, 544, 545). For example, the erythropoiesis-stimulating activity in mesangial cell-conditioned medium, previously reported by us (370) and confirmed by others (219), fails to compete with erythropoietin in immunological assays (unpublished observation). On the other hand, several of the tumor-derived cultures produce erythropoietin in large amounts but do so in a constitutive, apparently uncontrolled, manner (86, 278, 336, 635).

To establish a homogeneous cell culture system in which genuine erythropoietin is synthesized in a controlled manner, Goldberg et al. (236) screened multiple renal and hepatic cell lines. Of the renal glomerular or tubular cells tested, none produced measurable amounts of erythropoietin. Only two human hepatoma cell lines, Hep3B and HepG2, were capable of controlled erythropoietin gene expression as shown by bioassay, radioimmunoassay, and blot hybridization with erythropoietin DNA probes (236). Derived from liver biopsies of two children, the cells share morphologic and functional features with hepatocytes. In addition to plasmalike hepatic proteins of the adult, they produce α -fetoprotein like fetal liver (348).

The formation of erythropoietin in Hep3B and HepG2 cultures has been shown to rise greatly with cell density. When the O_2 concentration in the incubator was lowered from 21 to 2%, the degree of the synthesis of erythropoietin was enhanced at low but not at high cell density (236). Other investigators have confirmed the hypoxia-induced stimulation of the production of erythropoietin in HepG2 cultures at low cell density (654) and its inhibition at high cell density (486). The reason for this dependency on cell density has been clarified by microelectrode measurements of the O_2 tension directly in the cell layer. Within 1–2 h, the O_2 tension decreased to undetectable values (<1 mmHg) in confluent HepG2 monolayers maintained in the common culture dishes made of polystyrene (696). For a quantitative analysis of O_2 supply in tissue cultures, see reference 323. The problem of limited O_2 diffusion has been circumvented by shaking the cultures under gases containing O_2 at various concentrations. Assayed by competitive polymerase chain reaction with mutant competitor DNA, erythropoietin mRNA levels were 100-fold elevated when the O_2 concentration in the incubator was lowered from 21 to 1% (171). Studies with nuclear extracts from hypoxic Hep3B cells have provided evidence to assume that this increase in mRNA is at least partly due to increased erythropoietin gene transcription (94). Taken together, Hep3B and HepG2 cultures exhibit controlled, *i.e.*, O_2 dependent, synthesis of erythropoietin and give an appropriate *in vitro* system for study of the produc-

tion of the hormone, provided the O_2 supply is adjusted appropriately.

Using the Hep3B culture system, Goldberg et al. (234) have addressed the question as to the nature of the O_2 sensor in control of the production of erythropoietin. Because the formation of erythropoietin mRNA during hypoxic incubation was prevented by cycloheximide, it seems likely that protein synthesis is required before transcription of the erythropoietin gene is initiated. In addition, the production of erythropoietin in Hep3B cells was lowered in the presence of the heme synthesis-inhibiting compounds desferrioxamine and 4,6-dioxoheptanoic acid. On the basis of these findings the hypothesis has been developed that the biochemical substrate of the O_2 sensor is a heme protein and that ligand binding to its heme moiety influences the synthesis of erythropoietin (234). In support of this concept, the hypoxia-induced production of erythropoietin was inhibited when 10% CO was added to the incubation atmosphere. Carbon monoxide is known to bind to the ferrous iron in heme groups, resulting in an oxylike conformation.

In an attempt to identify the nature of the relevant hemoprotein, the production of erythropoietin was studied in HepG2 cultures treated with agents that interfere with microsomal mixed-functional oxidases [MFOs (172, 173)]. Composed of cytochrome *P*-450 and the flavoprotein NADPH-cytochrome *P*-450-oxidoreductase (*P*-450-reductase), this extramitochondrial hemoprotein system catalyzes the oxidation of its substrates by insertion of single atoms of oxygen derived from molecular O_2 . The rate of the production of erythropoietin increased in HepG2 cells following the addition of phenobarbital and 3-methylcholanthrene, which induce MFOs. The production of erythropoietin was also stimulated when compounds like cobaltous chloride or thyroid hormones were added that activate *P*-450-reductase alone. On the other hand, erythropoietin production was lowered by the MFO-inhibiting compounds diethyldithiocarbamate and cysteamine chloride (173). Known to reduce cytochrome *P*-450 levels in hepatocytes (621), interleukin-1 has been shown to suppress the synthesis of erythropoietin in HepG2 and Hep3B cells (174, 317a, 322; see sect. IXB). These findings have led to the hypothesis that the signal for the expression of erythropoietin mRNA could be provided by desoxygenated cytochrome (Fe^{2+}) *P*-450, which accumulates at low O_2 tension (or under the experimental condition of increased *P*-450-reductase activity). Similar to the liver, the kidney exhibits significant heme synthetic capacity and MFO activity (698), but its relevance to the renal production of erythropoietin has not been studied as yet.

Goldberg et al. (234) have pointed out that the reversibly O_2 -binding hemoproteins are more suitable in O_2 -sensing than O_2 -consuming redox systems. Indeed, inhibition of mitochondrial electron transport by cyanide failed to stimulate the formation of erythropoietin in HepG2 cultures (172). It has been shown earlier that neither cyanide nor moniodoacetic acid or fluoroacetate, which block glycolysis and citric acid cycle, respectively, influence the production of erythropoietin in normoxic or hypoxic animals (479, 481). In considering

the cellular redox state, it is also of note that the glutathione system is unlikely to play a major role in the control of the synthesis of erythropoietin. Its production was unaltered in HepG2 cultures with greatly reduced glutathione content following the addition of buthionine sulfoximine (172). This result is of interest in view of evidence suggesting that H_2O_2 enhances the production of erythropoietin in human renal carcinoma cultures (652), because reduced glutathione is a major intracellular H_2O_2 scavenger.

Fitting the hemoprotein concept, too, could be the previously unexplained mechanism (cf. Ref. 309) by which cobaltous ions stimulate the production of erythropoietin. Cobalt is not only a stimulus to microsomal *P*-450-reductase. Similar to nickel and manganese, it can substitute for ferrous iron in the center of the porphyrin ring of heme, which is so locked in the deoxy conformation. Indeed, cobaltous and nickel chloride greatly increased the synthesis of erythropoietin in Hep3B cultures at high O_2 tension but not in hypoxia (234). The action of cobalt was confirmed in HepG2 cultures (173, 236, 654). Cobalt-induced production of erythropoietin in the kidney and the liver has been shown earlier in experimental animals (38, 238, 445, 585) and in isolated organs (27, 67, 189, 191). Unlike hypoxia, cobalt exerts its effect on in vivo erythropoietin synthesis only after a lag of ~6 h. Nickel subsulfide must be injected directly into the kidney to stimulate the in vivo synthesis of erythropoietin (288).

The steady-state erythropoietin mRNA level in Hep3B cells increased >50-fold in response to hypoxia according to Northern blot analyses, whereas erythropoietin gene transcription increased only 10-fold in nuclear run-off experiments (235). Measurements of erythropoietin mRNA turnover in actinomycin D-treated Hep3B cells showed the half-life of the message to be markedly prolonged in hypoxic compared with normoxic cells. Thus, apart from stimulation of erythropoietin gene transcription, stabilization of the transcript appears to be another mechanism for hypoxia-induced synthesis of erythropoietin. Specific *trans*-acting mRNA-binding proteins are important in the posttranscriptional control of mRNA levels. In lysates of Hep3B cells, a cytosolic protein has been identified that bound specifically to a *cis*-element of the 3' untranslated region of erythropoietin mRNA (546a). This protein was present in both normoxic and hypoxic Hep3B cells. In addition, erythropoietin mRNA binding protein was detected in various murine tissues, including kidney, liver, brain, and spleen. Hypoxia-induced accumulation of this protein was seen only in spleen and brain (546a).

Whether second messenger molecules act on *trans*-protein factors has not been investigated with respect to the production of erythropoietin. However, cell culture studies have provided evidence that suggests that cyclic nucleotides and calcium-dependent protein kinases are involved in the synthetic pathway of erythropoietin. Adenosine 3',5'-cyclic monophosphate (cAMP) has been shown to enhance the release of erythropoietin in human renal carcinoma (271, 593), human hepatoma (654), and fetal mouse liver cultures (369). Adenosine 3',5'-

cyclic monophosphate is known to increase the blood level of erythropoietin when given to experimental animals (582). Guanosine 3',5'-cyclic monophosphate (cGMP) also enhanced the secretion of erythropoietin in human renal carcinoma cultures (653). In addition, erythropoietin production in these cells was stimulated by lowering the concentration of Ca^{2+} in the culture medium or by adding the calmodulin inhibitor trifluoperazine (473). The calcium entry blocking agent verapamil has been reported to increase the plasma erythropoietin level in hypoxic rats (420). However, other studies applying calmodulin antagonists were to the contrary, with trifluoperazine inhibiting erythropoietin production in HepG2 cells (unpublished observation) and with calmidazolium and W-7 inhibiting in isolated perfused rat kidneys (578a). One mechanism for the action of Ca^{2+} could be the stimulation of phospholipid-dependent protein kinases C. Long-term incubation of human renal carcinoma (270) and hepatoma (314a) cells with phorbol 12-myristate 13-acetate abolished production of erythropoietin. This effect of phorbol 12-myristate 13-acetate was associated with downregulation of a specific (α -type) protein kinase C isoenzyme in HepG2 cells (314a).

VI. PARACRINE AND ENDOCRINE MODULATORS OF ERYTHROPOIETIN PRODUCTION

A. Local Messengers

1. Prostanoids

The involvement of renal prostanoids in the production of erythropoietin was proposed by Fisher (186, 187). Hypoxia is a strong stimulus to the synthesis of prostaglandin (PG) E_2 and of prostacyclin in the kidney (319). The prostanoid synthesis-inhibiting drugs meclofenamate and indomethacin were shown to lower the increase of plasma erythropoietin in hypoxemic animals (466, 483) or following renal artery constriction (260, 465). The main renal prostaglandin PGE_2 , PGA_2 (259), and the prostaglandin precursor arachidonic acid (198) enhanced the release of erythropoietin in the programmed isolated perfused dog kidney. Prostanoids are thought to stimulate the renal secretion of erythropoietin by activating adenylate cyclase, thus increasing the concentration of cAMP intracellularly (187). The role of prostanoids in modulating the production of erythropoietin at extrarenal sites is less clear. Both the application of E- and A-type prostaglandins (477) and of indomethacin (208) resulted in increased plasma erythropoietin levels in nephrectomized rats exposed to hypoxia.

2. Adenosine

Adenosine has been proposed to stimulate the renal secretion of erythropoietin by A_2 receptor-mediated activation of protein kinase A (187). Actually, the depletion

of cellular ATP levels leads to an increased concentration of AMP, which is then metabolized to adenosine, inosine, and hypoxanthine. Adenosine was found to enhance the production of erythropoietin in experimental animals (582, 651), in isolated perfused rat kidneys (504), and in Hep3B cultures (473a). The adenosine antagonist theophylline has been applied recently to lower the plasma erythropoietin level in normal humans and, most importantly, in patients with erythrocytosis after renal transplantation (26). Still, the mechanism of action of theophylline is not fully understood, because this drug was shown earlier to enhance the plasma level of erythropoietin in hypoxemic animals (582).

3. Renin-angiotensin II

Renin and erythropoietin activities in blood may increase simultaneously (250), such as following a reduction of the renal blood flow. However, there does not appear to be a direct link between the release of renin and the synthesis of erythropoietin (2, 125, 435). The idea that erythropoietin and angiotensinogen are related molecules (221) has been disproven, since structural studies revealed no regions of homology in the amino acid sequences of the two glycoproteins (300). Renin (250, 251) and angiotensin II (193, 208, 251) at high concentrations stimulate renal and extrarenal production of erythropoietin, perhaps by causing ischemic hypoxia. In addition, there is evidence from clinical studies that treatment with angiotensin-converting enzyme-inhibiting drugs results in lowered plasma erythropoietin levels (220, 333).

B. Hypothalamus and Humoral Messengers

The hypothalamus was earlier proposed to influence the production of erythropoietin via humoral and neural routes (274). This concept has received less attention since O_2 -dependent erythropoietin production was demonstrated in isolated renal and hepatic tissue. However, if there were only local control, some phenomena would remain unexplained. One of these is the circadian rhythm of the erythropoietin level in blood (98, 442, 685). The reported 60% decrease between midnight and 4 A.M. in humans implicates that the production of the hormone almost ceases after midnight, because the half-life of circulating erythropoietin in humans is several hours (see sect. VII B). Another finding at odds with purely local intrarenal control is the slight increase in plasma erythropoietin occurring when the O_2 supply to the kidneys alone is reduced (498, 499). Hence it is worthwhile recalling some of the studies suggesting a regulatory, or at least permissive, role for the hypothalamic-hypophysial system.

Electrical stimulation of the hypothalamus induced a rise in plasma erythropoietin activity in rabbits (274) and monkeys (449). Reticulocytosis slowed down in hypoxic rabbits with lesions in the posterior, middle, and ventral hypothalamus but not in the anterior hypothalamus.

mus (273). Anemia is known to develop in hypophysectomized animals and in humans suffering from panhypopituitarism. Because plasma erythropoietin still increases to some extent in hypophysectomized anemic humans, a synergistic action of the pituitary gland has been assumed (467). Hypophysectomy greatly reduced plasma erythropoietin in hypoxemic rats (275, 514) and rabbits (275). The hypophysial influence on the production of erythropoietin in hypoxia is apparently mediated by the concerted action of several hormones, including adrenocorticotrophic hormone (ACTH), GH, thyroid hormones, and sex steroids. In addition, these hormones are important in the control of the cellular metabolism and thus the rate of O_2 consumption.

1. Adenohypophysial hormones

The erythropoiesis-stimulating effect of ACTH given to mice was abolished by the application of erythropoietin antiserum (192, 517) and was mimicked only in part by the administration of cortisol (517). High doses of glucocorticoids were found to lower the production of erythropoietin in hypoxic mice (247). Like ACTH, GH appears to be permissive for erythropoietin production, because the administration of GH resulted in increased plasma erythropoietin activity in hypophysectomized rats (428, 516). On the other hand, the application of recombinant human erythropoietin has been reported to induce a lowering of the basal plasma concentrations of ACTH and GH in renal-insufficient patients (351). The mechanism of this action of erythropoietin has remained obscure.

2. Thyroid hormones

The thyroid hormones, triiodothyronine (T_3) or thyroxine (T_4), have been shown to stimulate the production of erythropoietin in experimental animals (192, 519) and in hepatoma cell cultures (173). The suppressed erythropoietin response in fasting animals (18, 19, 210, 315, 562) was restored by the application of T_3 (77). Because the cytochrome *P*-450 system has been assigned a key role in erythropoietin production (173; see sect. vB), of interest are studies demonstrating a decrease in hepatic activities of some cytochrome *P*-450 isozymes and of *P*-450-reductase in hypophysectomized rats (676). The activity of the cytochrome *P*-450 system was substantially restored following the application of T_4 (676), as is the erythropoietin response to hypoxia.

3. Sex steroids

Renal and extrarenal erythropoietin production in response to acute hypoxic stress is lower in female than in male rats (206, 671). It has been reported that estrogenic steroids lower the hypoxia-induced production of erythropoietin in rats (515). On the other hand, androgenic steroids were shown to increase the production of

erythropoietin in experimental animals (192, 246, 428); in programmed isolated perfused dog kidneys (399, 506), although not in normal perfused kidneys (246); and in fetal liver cell cultures (711). The effect of testosterone decreased in rats after 2 wk of treatment (246). The transient stimulation of the production of erythropoietin by androgen may provide an explanation for the fact that neither normal (95, 320, 401, 440, 461, 535) nor chronically anemic (320) men and women differ with respect to their plasma erythropoietin level. In addition, the application of 19-nortestosterone decanoate failed to enhance the level of immunoreactive erythropoietin in normal men (440). In all likelihood, the higher blood hemoglobin concentration of males compared with females mainly results from androgenic stimulation and estrogenic inhibition of the proliferation of erythrocytic progenitors directly in bone marrow (5, 131, 188, 324).

4. Catecholamines

Activation of the autonomous nervous system has been considered another means of increasing the production of erythropoietin in hypoxia. Plasma erythropoietin levels were lowered in hypoxemic rats immediately after the splanchnic nerves were cut but normalized 1 wk thereafter (42). It is clear from experience with kidney-transplant patients that renal nerves are not essential for production of erythropoietin. It should be noted, however, that the blood level of erythropoietin was transiently lowered in hypoxemic rabbits after bilateral renal denervation (177). Cholinergically mediated events cannot be excluded, because the application of methylatropine reduced the erythropoietin response in hypoxemic dogs (606). However, most studies have focused on adrenergic effects. The capacity to produce erythropoietin was blunted in hypoxic rabbits pretreated with reserpine (180). Because the β_2 -adrenergic blocking agent butoxamine inhibited while the α -adrenergic antagonist phenoxybenzamine enhanced the erythropoietin level, the sympathetic stimulation of the production of erythropoietin is thought to involve a β_2 -adrenergic mechanism (180, 181). Indeed, single injections of β_2 -adrenergic agonists such as albuterol in high doses increased the plasma erythropoietin activity in experimental animals (178–180). Albuterol stimulated the formation of erythropoietin in programmed isolated perfused dog kidneys (313, 529). The nonspecific β -adrenergic agonist isoproterenol increased the occurrence of erythropoietic activity in fetal mouse liver cell cultures (369). β -Adrenergic activation of the secretion of erythropoietin is thought to be mediated by cAMP-dependent protein kinase A (179, 187, 369).

In the day-to-day control of red blood cell production of normoxic individuals, adrenergic stimulation appears to play a minor role. Long-term treatment with high doses of albuterol resulted in elevated red blood cell mass in hypoxic rabbits (312) and anemic mice (310), but no such effect was seen in normoxic animals. Neither does physical stress-induced activation of the sympathetic nervous system cause a significant increase in plasma erythropoietin in human athletes (543, 578).

VII. KINETICS OF ERYTHROPOIETIN IN PLASMA

A. *Dynamic of Formation*

A measurable increase in plasma erythropoietin has been documented as early as 1–2 h after the initiation of anemia or hypobaric hypoxia in humans (136) and in rats (443, 581, 586). Hypoxic periods of 15–30 min are sufficient to induce a transient rise in plasma erythropoietin in rodents (71, 139, 478). Following acute hemorrhage in rats (157, 318, 426, 444, 447) and rabbits (475), plasma erythropoietin increases for 12–48 h concomitantly with the fall in the hemoglobin concentration and then drops despite persisting anemia. Exposure to hypoxic hypoxia also induces initially an excessive rise in plasma erythropoietin. Peak values were detected after 6 h to 3 days in humans (1, 435), dogs (629), and rodents (1, 75, 210, 307, 444, 583, 587, 600, 615, 710). In addition, a transient increase in plasma erythropoietin was measured in anephric rats following bleeding or hypoxia exposure (157).

Thus the erythropoietin response to an acute hypoxic stress is dynamic, i.e., initially very great and then more moderate. The major increase in the concentration of red blood cells occurs only after the decline in plasma erythropoietin (210, 307, 587). Of course, erythropoietin levels in uncomplicated chronic anemia or hypoxemia are still higher than in normoxia and are, in fact, inversely correlated with the degree of the lowering of the O_2 availability. Whether the initial overshoot in erythropoietin activity is important to trigger immature erythrocytic progenitors into proliferation is not known, but it is clear that the lower levels with continued hypoxia suffice to maintain an enhanced rate of erythropoiesis.

Serial measurements of the renal erythropoietin content in rats exposed to hypobaric hypoxia have shown that the transient increase of erythropoietin in blood is preceded by a rise in the kidney (75, 307). The maximum was reached in the kidney after 6 h. The renal erythropoietin content then decreased despite continued hypoxia. These findings provided evidence that the changes in the plasma level of erythropoietin during hypoxia reflect changes in its renal synthesis and that a decrease in erythropoietin production rather than an increase in its degradation is responsible for the decline in plasma erythropoietin levels occurring after 1–3 days of continuous hypoxic stress (307). Northern blot analyses confirmed this contention. Renal erythropoietin mRNA was shown to be maximally expressed 4–8 h after the induction of anemia in mice and to drop on the following days before the recovery of hematocrit (52, 359).

Several studies have addressed the question as to why the production of erythropoietin slows down before an effective increase in the concentration of red blood cells even when systemic hypoxia persists. Circulating erythropoietin per se does not exert feedback inhibition at the sites of its synthesis, as the injection of large doses of the hormone in rats did not suppress the hypoxia-induced increase in plasma erythropoietin (137,

205). An increase in ventilation, enabled by renal compensation of respiratory alkalosis, may contribute to the lowering of erythropoietin production in the course of hypoxic hypoxia (92, 444, 583, 695). Some authors have related the dynamic pattern of erythropoietin production to the accompanying changes in the O_2 affinity of the blood following the induction of hypoxemia (446, 695) or anemia (447). The O_2 affinity of the blood increases initially in parallel with red blood cell pH. It returns toward normal only after several hours. Associated with an increase in red blood cell 2,3-bisphosphoglycerate levels, the O_2 affinity of the blood may later decrease below normal, which facilitates the unloading of O_2 from the blood. When these adaptive processes were reversed by exposure of rats to discontinuous hypoxic hypoxia with intermittent periods of normoxia lasting 1 day, the rise of the renal (307) and plasma (210) erythropoietin activities was similar to that of rats not previously subjected to hypoxia. Because this was also seen in rats deprived of food during the normoxic intervals (210, 315), the low levels of erythropoietin during continuous hypoxia are not primarily caused by reduced food intake as suggested (134).

B. *Metabolism*

Like other glycoproteins, erythropoietin is cleared rather slowly from the blood. It is mainly the terminal sialic acid residues that prevent the hormone from galactose receptor binding, internalization, and degradation in the liver (127, 215, 229, 240, 397, 609). The metabolic fate of endogenous and exogenous natural erythropoietin has been investigated in humans and in animals. Embury et al. (148) calculated half-lives of endogenous erythropoietin as being between 1.5 and 2.9 h in three anemic sickle cell patients whose elevated plasma erythropoietin values fell to normal within 2 days during therapeutic inhalation of pure O_2 . Following exposure of humans to hypobaric hypoxia, plasma erythropoietin was calculated to decline, with an average half-life of 5.2 h (136). In rats previously exposed to hypoxic hypoxia, plasma erythropoietin decreased with a half-life of ~2 h (123, 472). However, these data may be overestimates, because the production of erythropoietin does not cease immediately at the end of a hypoxic stimulation but continues for ~2 h (71, 205, 581, 583, 586).

Indeed, the half-life of intravenously applied homologous erythropoietin was calculated to be only 1.5 h in rats (610). Estimates in mice have ranged from 2 to 3 h (1, 448). Eight to 10 h have been reported for homologous erythropoietin in sheep (455) and for human erythropoietin in dogs (213). In anephric animals, elimination of erythropoietin was retarded in some studies (123, 149, 213) but not in all (455, 610). In rats given radiolabeled pure human urinary erythropoietin, 32% was removed by the kidneys, but only 7% was recovered in the urine (149).

Of clinical interest are the pharmacokinetics of the recombinant product, which differs from the natural hormone to some extent in sialylation (560, 632). When

administered as an intravenous bolus, recombinant erythropoietin is cleared from plasma in an exponential way. Half-life estimates have ranged from 2 to 13 h both in normal subjects and in patients with renal insufficiency, with half-life tending to increase as a function of dose (101, 196, 387, 398, 425, 485, 557). Plasma erythropoietin clearance is <10 ml/min. Estimates of the apparent volume of distribution of recombinant erythropoietin in humans have ranged from 30 to 100 ml/kg body wt, depending on plasma volume (101, 196, 387, 398, 485, 557).

According to a long-established hypothesis (615), catabolism of erythropoietin by utilization in erythropoietic tissues could be important in addition to hepatic metabolism and renal clearance. Indeed, the plasma erythropoietin concentration is not only inversely correlated with O₂ availability but is also dependent on the proliferative activity of the erythropoietic tissue. Patients with myeloid hypoplasia have relatively high levels of bioactive (116, 276, 326, 475, 510, 631) and immunoreactive (321, 422) erythropoietin in relation to the degree of their anemia. The serum erythropoietin concentration increased abruptly in leukemic patients 1–2 days after cytotoxic treatment was started (3, 47, 284, 521). On the other hand, the erythropoietin level dropped markedly in leukemic patients when erythropoiesis recovered in the early stage of remission, but anemia was still persistent (321). Additionally, laboratory studies have revealed unusually high erythropoietin levels in anemic mice with a congenital hemopoietic stem cell defect (209, 552) and in hypoxemic mice or rats with marrow hypoplasia induced by X-irradiation or the injection of 5-fluorouracil (28, 210). The estimates of erythropoietin clearance in animals with marrow hypoplasia compared with normals are contradictory. Both increased half-lives of erythropoietin in association with marrow hypoplasia (455, 552), and no differences (57, 209, 210, 472) have been reported. Neither were previous attempts conclusive in demonstrating differences in the half-life of the hormone in patients with renal failure before and after activation of erythropoiesis by recombinant erythropoietin therapy (101, 387, 485, 557).

In summary, although plasma erythropoietin is abnormally high in subjects with erythrocytic hypoplasia, there is a lack of experimental evidence indicating that changes in the rate of the uptake of erythropoietin by its target cells are important in the control of the plasma level of the hormone. Alternatively, humoral factors derived from proliferating erythrocytic progenitors could lower by feedback inhibition the production of erythropoietin (28, 210, 321, 422).

VIII. MECHANISM OF ACTION OF ERYTHROPOIETIN

A. *Erythropoietin-Responsive Cells*

New blood cells are generated continuously from proliferative progenitors in the hemopoietic organs. Three main developmental stages can be distinguished in the sequence of cell division, differentiation, and mat-

uration. First are self-renewing pluripotent stem cells capable of producing progeny that are committed to either erythrocytic, megakaryocytic, granulocytic, or monocytic differentiation. Controlled by a variety of growth factors, including erythropoietin, terminally differentiated morphologically identifiable blood cell precursors emerge.

Studies of the effects of purified recombinant erythropoietin in clonal assay systems using hemopoietic cells in culture have confirmed the concept that the erythropoietin-responsive compartment spans mainly erythroid-committed progenitors and early erythroblasts. Following the pluripotent hemopoietic stem cell and the mixed-lineage myeloid progenitor [colony-forming unit-mixed (CFU-MIX or CFU-GEMM)], the most primitive erythrocytic progenitor is the burst-forming unit-erythroid (BFU-E), which gives rise to large multiclustered bursts of hemoglobin-synthesizing progeny when cultured with high doses of erythropoietin (23, 121, 225, 298). The *in vitro* growth of BFU-Es requires cytokines having burst-promoting activity (BPA), such as interleukin-3 or GM-CSF (241, 433, 434, 459, 496, 566, 618). *In vivo* only a small portion of BFU-Es is in active cell cycle (255, 296, 537). Erythropoietin appears to trigger its target cells into S phase (646). There are at least two subclasses of BFU-Es, an early and a more differentiated subclass with increased sensitivity to erythropoietin (135, 254, 255, 334).

By proliferation and differentiation, BFU-Es produce cells that resemble proerythroblasts. Called colony-forming units-erythroid (CFU-E), these give rise to small clusters of hemoglobinized cells when cultured with erythropoietin (424). The formation of red blood cell membrane proteins increases before the onset of the synthesis of hemoglobin (642). The CFU-Es are very sensitive to erythropoietin. Moreover, they have an absolute requirement for the hormone for colony formation and terminal differentiation. *In vivo*, high numbers of CFU-Es are in active cell cycle, and their percentage increases further with erythropoietin (255, 537). The CFU-Es are considered the main target cells of erythropoietin. Stimulation of erythropoiesis is effected by amplification of the pool of CFU-Es. Recent evidence suggests that CFU-Es lack the potential for self-replication. Hence it has been proposed that an increased influx from the pool of BFU-Es is responsible for the expansion of the compartment of late erythrocytic progenitors during rapid erythroid regeneration (656). Plasma erythropoietin fluctuations have been correlated with changes in the size of the CFU-E compartment. The number of CFU-Es was found to be reduced in plethora and to be increased in anemia (8, 255, 296, 512). As reviewed earlier (309), the effect of erythropoietin on the growth of CFU-Es is augmented by several other hormones, including androgens, thyroid hormones, somatomedins, and catecholamines.

In addition to stimulating the proliferation and differentiation of the erythrocytic progenitors, erythropoietin activates the mitotic division of proerythroblasts and basophilic erythroblasts (232, 650), and it accelerates the release of reticulocytes from the marrow (502).

Maximum reticulocytosis occurs usually 3–4 days after an acute increase in plasma erythropoietin.

Biochemical analyses of the action of erythropoietin in the terminal differentiation of CFU-Es to reticulocytes have been hampered by the lack of homogenous populations of erythrocytic progenitors. Therefore, as a model system, erythropoietic tissues were infected *in vivo* or *in vitro* with Friend virus (FVA) of the anemia-inducing strain (53, 569). The FVA-infected cells can differentiate without erythropoietin until they reach a proerythroblast-like state, where they are arrested and eventually die (353, 354). On the addition of erythropoietin, synchronous maturation of FVA-infected proerythroblasts is initiated with nuclear condensation, globin gene transcription (53), increased expression of transferrin receptors (570), and synthesis of hemoglobin and other red blood cell-specific proteins (355, 356).

The action of erythropoietin is not restricted absolutely to the erythrocytic pathway. Pure recombinant human erythropoietin has been shown to enhance the *in vitro* growth of CFU-MIX (225). In uremic humans treated with erythropoietin for 3 wk, not only the numbers of CFU-Es and BFU-Es but also those of CFU-MIXs and of CFU-megakaryocytic (MKs) were significantly increased in the bone marrow (120, 617). Increased counts of circulating BFU-Es and CFU-MIXs have also been reported (224). The reduced number of marrow BFU-Es in humans treated with erythropoietin in the long term has been related to the permanent loss of this progenitor type in association with an insufficient replacement from the pool of pluripotent stem cells (537).

Whether erythropoietin alone is capable of stimulating *in vitro* megakaryopoiesis is unclear (64, 88, 225, 261, 299, 555, 637). Recombinant human erythropoietin increased the number and size of megakaryocytic colonies in the presence of interleukin-3 or GM-CSF in cultures from human (64, 119, 637) or mouse marrow (88). Specific, high-affinity binding sites for erythropoietin were demonstrated on rat and mouse megakaryocytes by autoradiographic analyses (199). Single injections of erythropoietin failed to stimulate megakaryocytopoiesis in normal mice, as judged from determinations of platelet count, platelet size, and [^{75}Se]selenomethionine incorporation into platelets (261). A transient increase in the number of circulating platelets was observed in splenectomized mice (648) and in normal rats (34) on days 4 and 5 after the repeated injection of high doses of recombinant erythropoietin. The chronic use of recombinant erythropoietin in uremic patients does not induce thrombocytosis (162, 387, 458, 549), although an increase by 10–20% in platelet number may occur (160, 243, 458, 658, 660). Earlier studies showed platelet production to be unchanged in hypoxemic mice with elevated endogenous levels of erythropoietin (169). Moreover, platelet counts were lowered in transgenic mice permanently expressing the human erythropoietin gene (589). Taken together, erythropoietin appears to give little effect to the number of platelets in the blood.

An elevated number of granulocytic-monocytic progenitors (CFU-GM) was found in the marrow of hu-

mans during erythropoietin therapy (120). Circulating CFU-GMs increased in one study (613) but not in another (225). Conflicting data also exist with respect to the *in vitro* action of recombinant human erythropoietin on CFU-GM growth, with reports of no effect (88, 119, 225) or inhibition (87, 350). The administration of high doses of erythropoietin had no effect on the number of circulating neutrophilic granulocytes, monocytes, or lymphocytes in young rats (350). Neither were leukocyte counts abnormal in transgenic mice carrying an active additional human erythropoietin gene (589).

In conclusion, erythropoietin is a relatively restricted hemopoietic hormone. It enhances specifically the growth of erythroblasts from the erythrocytic progenitors, which include primitive BFU-Es, differentiated BFU-Es, and CFU-Es. Additionally, erythropoietin-induced activation of the erythrocytic pathway may exert a feedback stimulation of the pool of the less differentiated stem cells. Thereby, and in concert with other cytokines, erythropoietin could play a modulating role in the control of the megakaryocytic and granulocytic-monocytic pathways. Perhaps the use of pure recombinant erythropoietin will uncover novel targets of the hormone. Indeed, recent observations suggest it enhances the *in vitro* migration and proliferation of endothelial cells (17).

B. Erythropoietin Receptor

Similar to other protein hormones, erythropoietin acts by binding to specific cell membrane receptors. Their existence was first demonstrated in studies using tritiated purified human urinary erythropoietin and spleen cells from FVA-infected mice (361). The amino acids 111–118 of erythropoietin were thought to bind to the receptor, since antibodies against synthetic peptides spanning residues 99–118 or 111–129 inhibited the biological action of the hormone, while antibodies to other regions were not inhibitory (626). However, these findings are not conclusive, because the action of antibodies is not restricted to the specific epitopes of an antigen. Structure-function relationship studies with scanning deletion mutants of erythropoietin revealed full biological activity of mutant $\Delta 111$ –119. In contrast, deletions within the four helical domains resulted in an unstable product without biological activity (49a). In addition, oligosaccharide trimming investigations have shown the trimannosyl core of the *N*-linked sugar chains to be important in maintaining an active conformation of the hormone (633).

Insight into the erythropoietin receptor has been made possible by the development of techniques to isolate pure populations of erythrocytic progenitors (488, 566, 603) and to radioiodinate (571, 641) or biotinylate (692) erythropoietin without loss of biological activity and by the availability of recombinant erythropoietin. Iodinated erythropoietin was shown to bind to a high- and to a low-affinity type of murine receptor (214, 563, 571, 572). Hormone binding was rapidly followed by internalization and subsequent degradation of the hormone (571, 641).

Two classes of erythropoietin receptors were also demonstrated on CFU-Es isolated from human blood, with dissociation constants of 0.10 and 0.57 nM, respectively, and a total of 1,050 binding sites/cell (567). As in the murine system, internalization and degradation occurred. Because proteolytic digestion of the two human receptor species (85- and 100-kDa erythropoietin cross-linked proteins) resulted in identical fragments and because the addition of protease inhibitors during membrane isolation prevented the appearance of the smaller component, the 100-kDa complex appears to correspond to the physiological receptor (568). The presence of single or dual high- or low-affinity erythropoietin binding sites has been demonstrated in other mammalian systems (cf. Ref. 110), including human (59, 548), rodent (11, 59, 377, 408, 409, 412, 463, 556, 641, 692), and hamster (56) erythrocytic; rodent megakaryocytic (199); rodent placenta (573); and human myeloma cells (492). Scatchard and autoradiographic analyses confirmed the dependence of erythropoietin binding capacity on the stage of erythrocytic differentiation and maturation. Primitive BFU-Es isolated from human blood had much fewer erythropoietin receptors than CFU-Es, but their receptor density increased with differentiation (566). Proerythroblasts from rat bone marrow were found to have 390 receptors/cell, twice as many as basophilic or polychromatophilic erythroblasts (11).

The cDNAs for the murine and the human erythropoietin receptors have recently been isolated and characterized. The murine erythropoietin receptor gene comprises ~5 kb and is present in a single copy per haploid genome (109, 366, 386, 705). The membrane-spanning domain was shown to be encoded by a single exon. The human homologue of the murine erythropoietin receptor has been isolated from erythroleukemia cells and fetal liver, and its coding sequence has been determined (332, 689). The human gene has been localized to chromosome 19p by *in situ* hybridization (65, 689). The predicted sequence of 508 amino acids of the receptor protein shows 82% homology to the murine erythropoietin receptor (332, 689). Heterologous expression of the human cDNA in COS cells yielded a protein of ~66 kDa (332). The reason for the difference in molecular mass of the cDNA-derived erythropoietin receptor and the receptors identified by cross-linking is not understood (289, 411). On the basis of solubilization studies yielding nondenatured receptor complexes, it has been proposed recently that the genuine erythropoietin receptor is a multimeric complex of disulfide-linked proteins, having an estimated molecular mass of ~300 kDa (410).

There is striking homology of the extracellular domains of the receptors for erythropoietin, interleukin-2 β -chain, interleukin-6, GH, and prolactin. The evolutionary and functional meanings of this novel family of growth factor receptors have been discussed in an interesting article by Bazan (31). The understanding of the structure of the erythropoietin receptor is of great interest with a view not only to the physiological events of erythrocytic differentiation but also to disorders with increased sensitivity to erythropoietin, such as polycy-

themia vera. Yoshimura et al. (704) have described a single point mutation in the exoplasmic domain of the erythropoietin receptor that induced erythropoietin-independent cell growth and tumorigenesis in hemopoietic Ba/F3 cells.

C. Signal Transduction

When the erythropoietin-receptor complex is internalized, some kind of signal must be transferred to the nucleus. In rat bone marrow cultures treated with pure human urinary erythropoietin for 60 min, a cytoplasmic protein was formed that enhanced transcription in isolated marrow nuclei (679). The nature of this protein mediator has remained unknown. There is no tyrosine kinase motif in the cytoplasmic domains of the erythropoietin receptor (109). In addition, neither cAMP nor cGMP seem to act as second messengers, although they play a modulating role in erythrocytic differentiation and maturation (51, 252, 647, 682). Lipoxygenase products of arachidonic acid metabolism have been considered essential for the differentiation of erythrocytic progenitors, because the selective lipoxygenase inhibitor, BW755C, diminished the erythropoietin-induced growth of erythroid colonies in fetal mouse liver cell cultures (32). However, clear proof is missing that eicosanoids are mediators of the effect of erythropoietin.

Inconclusive, too, is the knowledge of the role of Ca^{2+} in the action of erythropoietin. In the absence of calcium, erythropoietin suppressed the phosphorylation of a prominent 43-kDa protein in the membranes of murine erythrocytic tissue (85). On the other hand, the *in vitro* growth of murine CFU-Es was enhanced by the ionophore A23187 and was inhibited by the calcium chelator ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) (452). The proliferation, but not the differentiation, of murine erythroleukemia cells was blocked by EGTA (216). Some investigators have considered the inhibition by calcium-chelating agents to be a nonspecific (51) or toxic effect (293), as it was not restored by the addition of excessive Ca^{2+} . The effects of erythropoietin on the survival and differentiation of proerythroblasts from rabbit bone marrow could not be mimicked by artificially raising intracellular Ca^{2+} levels (51). Erythropoietin was shown to rapidly stimulate the uptake of $^{45}\text{Ca}^{2+}$ into cultured FVA-infected mouse bone marrow cells (569). Recombinant erythropoietin increased the Ca^{2+} level in human bone marrow mononuclear cells within a few seconds, as measured with fluorescent indicators (456). However, studies failed to obtain a fluorescence signal in erythropoietin-treated CFU-Es from murine spleen (293). Fluorescence microscopic digital video imaging showed recombinant erythropoietin and GM-CSF to induce a transient rise in intracellular free calcium in single BFU-E-derived erythroblasts cultured from fetal or adult human blood (436, 438). This effect required no extracellular Ca^{2+} . Hence stimulation of membrane phosphoinositide turnover with a resultant intracellular mobilization of Ca^{2+} is most likely (438). Because

pertussis toxin proved to block the Ca^{2+} increase in erythroblasts treated with erythropoietin (437), the mobilization of Ca^{2+} appears to be mediated by a pertussis toxin-sensitive GTP-binding protein. In addition, the free Ca^{2+} concentration was higher in the nucleus than in the cytoplasm of erythropoietin-stimulated human erythroblasts, indicating that intranuclear Ca^{2+} -sensitive enzymes could be involved in erythrocytic differentiation and gene transcription (703). Indeed, erythropoietin has been shown to induce protein phosphorylation in nuclei isolated from murine fetal liver cells (402). This effect could be blocked by inhibitors of protein kinases C. On the basis of these findings, and analogous to recent reports of nuclear protein kinase C activation by prolactin, a novel intracellular messenger system has been proposed for the action of erythropoietin, involving protein kinase C activation and protein phosphorylation in the nucleus of the target cells (402).

IX. PATHOPHYSIOLOGY OF PRODUCTION OF ERYTHROPOIETIN

A. Overproduction

Secondary erythrocytosis results from excessive production of erythropoietin. It may be induced by hypoxia or arise autonomously. In contrast, polycythemia vera is caused by erythropoietin-independent growth of erythrocytic progenitors from abnormal stem cell clones (81, 107, 365, 536), and afflicted patients have normal or lowered erythropoietin levels (46, 100, 117, 154, 156, 227, 349). However, single measurements of the serum erythropoietin concentration in individual subjects are of limited diagnostic value, as they do not enable one to discriminate clearly between the two forms of erythrocytosis (100).

1. In association with tissue hypoxia

With peripheral O_2 delivery being the main regulatory parameter, the production of erythropoietin increases at lowered arterial O_2 tension. Both natives and visitors at altitude exhibit erythrocytic hyperplasia of their bone marrow, erythrocytosis, and increased blood hemoglobin concentration (383, 430). Their red blood cell mass is inversely correlated with the arterial O_2 saturation. Even though the action of erythropoietin is protective against anemia, its benefits in terms of improved O_2 transport are outweighed by rheological and hemodynamic disadvantages in hypoxemia (142, 153, 640). Peripheral resistance and work load of the heart are increased. In particular, blood flow is obstructed in the lungs, where hypoxemia causes vasoconstriction. Erythrocytosis, congestive heart failure, and dyspnea are the most serious manifestations of chronic mountain sickness. As in polycythemia vera, venesection can be beneficial in patients with secondary erythrocytosis.

In contrast with altitude hypoxemia, chronic respi-

ratory failure at sea level seldom leads to severe erythrocytosis (210). In addition, there is no clear relationship between the red blood cell mass and the arterial O_2 tension and saturation. The development of erythrocytosis in chronic obstructive airways disease increases the risk of cor pulmonale (142). The percentage of patients reported with elevated plasma erythropoietin has varied between 21 and 85% (265, 442, 677), probably depending on the severity of hypoxemia. Intermittent nocturnal hypoxia in patients with obstructive sleep apnea is not accompanied by elevated serum erythropoietin or erythrocytosis (423).

The missing increase in the plasma erythropoietin level in chronic respiratory failure (200, 265) could be related to the associated retention of carbon dioxide. Hypercapnia is known to suppress the production of erythropoietin in experimental animals (see sect. vA). In addition, inflammation in the bronchial tree may partly account for the blunted erythropoietin response, as chronic inflammatory processes appear to diminish the production of the hormone (see sect. ixB). Indeed, in a study of chronically hypoxemic patients with cystic fibrosis, plasma erythropoietin levels were not elevated above normal, although one-third of the afflicted persons were anemic (664).

Erythrocytosis secondary to lowered arterial O_2 tension usually develops in patients with severe cyanotic congenital heart disease (230, 267, 649). Because the reduced O_2 saturation is offset by the increase in O_2 carrying capacity, blood erythropoietin values are often only slightly higher than normal (267, 325). This relationship may also hold true for other types of secondary erythrocytosis.

Hypoxia-induced erythrocytosis occurs as an inherited disorder in association with mutant hemoglobins or red blood cell 2,3-bisphosphoglycerate deficiency causing high blood O_2 affinity (7). In rare cases, secondary erythrocytosis is due to renal artery stenosis (cf. Ref. 264). Elevated plasma erythropoietin without manifest erythrocytosis was observed in subjects with congestive heart failure, and this elevation was abolished by treatment with the angiotensin-converting enzyme-inhibiting drug, enalapril (220).

Single cases have been reported of familial erythrocytosis resulting from elevated blood levels of erythropoietin in the absence of recognizable abnormalities in systemic or renal O_2 supply (112, 124, 365, 681). The inverse relationship between erythropoietin levels and hematocrit was maintained in some of the affected individuals (365, 681) but not in all (124).

Erythrocytosis develops in 8–18% of the recipients of kidney transplants. It is usually transient (26, 40, 282, 622, 638; for summaries see Refs. 683, 697). This phenomenon has been explained by an increased sensitivity and proliferative activity of erythrocytic progenitors (282, 375, 612). Other investigators have proposed that excessive stimulation of erythropoiesis in renal transplant recipients could be due to defective feedback inhibition of the production of erythropoietin either in the remaining native kidneys or in the transplanted kidney (10, 106, 638).

2. In association with tumors

Certain tumors cause erythrocytosis secondary to the production of erythropoietin by the neoplastic cells. The incidence of paraneoplastic erythropoietin synthesis is not exactly known, as it can be masked by anemia. Patients with malignancies are generally anemic because of acute or chronic bleeding, increased hemolysis, lack of iron and vitamins, and bone marrow suppression by inflammatory cytokines, cytotoxic drugs, and radiotherapy (329).

In a review of 340 cases of erythrocytosis associated with tumors, 35% of the patients had renal carcinomas (277). Erythrocytosis was demonstrated in 3% of the patients with renal carcinomas (340, 668). Thorling (639) measured increased plasma erythropoietin bioactivity in 19 of 25 patients with renal carcinoma-associated erythrocytosis. In contrast with our own much lower immunoassay estimates (unpublished observations), it has been reported that plasma erythropoietin bioactivity is elevated in 63% of all patients with renal carcinomas (620). This elevation was found to be independent of hematocrit and blood hemoglobin concentration (620). Blood levels of erythropoietin were also shown to be raised in 60–70% of the patients with Wilms' tumor (343, 468), with a much lower incidence of erythrocytosis (468, 605). Perhaps arising from pressure-induced ischemia, benign renal tumors may also induce erythropoietin production and erythrocytosis (84, 222, 277, 467, 590). By *in situ* hybridization, erythropoietin mRNA was localized in the cyst walls of the kidneys of patients with autosomal polycystic renal disease (141).

Of 340 cases with tumor-associated erythrocytosis, 19% suffered from hepatomas (277). Erythrocytosis was observed in 4–12% of liver carcinoma patients (344, 417), with high plasma erythropoietin bioactivity in ~70% of these (222, 277, 417). Immunoreactive erythropoietin was found elevated in 23% of the nonanemic patients with liver carcinoma (344).

Of the ectopic sites of erythropoietin production, first to be noted are cerebellar hemangioblastomas present in 20% of all patients with tumor-associated erythrocytosis (277, 306). Plasma erythropoietin activity is not usually elevated in this disease (285, 305, 484). By immunohistochemistry, erythropoietin was localized in scattered small granular cells of hemangioblastomas (49). On the basis of bioassay measurements, other sites of paraneoplastic erythropoietin synthesis have included the adrenal gland (668) and smooth muscle tumors such as uterine fibromyomas (277, 668) and leiomyomas (146, 212). Measured by hemagglutination inhibition, a case of erythropoietin production by atrial myxoma has been reported (69), but the validity of that assay system was not generally accepted (309).

Difficult to answer is the question as to whether measurements of plasma erythropoietin are useful in the diagnosis and follow-up of malignant diseases associated with autonomous erythropoietin production. Overall, attempts have failed to correlate the plasma erythropoietin level with tumor grade or stage in patients suffering from renal carcinoma (620). In some

cases of renal carcinoma (371) and Wilms' tumor (468), plasma erythropoietin, hematocrit, and blood hemoglobin concentration normalized after the tumor-bearing kidney was removed and later increased again heralding tumor recurrence. Measurements of immunoreactive erythropoietin were found of little diagnostic aid in von Hippel-Lindau syndrome, which often presents with renal carcinoma or cysts, cerebellar hemangioblastoma, and pheochromocytoma (484). In patients with hemangioblastomas, the assay of erythropoietin in the cerebrospinal fluid appeared to be more reliable, yielding high values despite low plasma erythropoietin (305).

B. Deficiency

1. Anemia of renal failure

Erythropoietin deficiency is the primary cause of the anemia in chronic renal failure. Other pathogenetic factors of varying importance include inhibition of marrow erythropoiesis by humoral toxins (201, 217, 419, 421, 670) and paracrine cytokines (376), bleeding, shortening of red blood cell survival, and malnutrition (547). The hypoproliferative anemia associated with uremia is usually normochromic and normocytic. Its severity increases with the impairment of renal function regardless of the underlying disease (82, 508). As a rule, significant anemia develops when the glomerular filtration rate falls below 40 to 20 ml/min and 1.73 m² (82, 118, 419, 527). Switching from hemodialysis to continuous ambulatory peritoneal dialysis occasionally improves the anemia (83, 708). Still, almost all of the patients in the later stages of renal failure were anemic until recombinant human erythropoietin became available for therapy.

The inverse relationship between plasma erythropoietin activity and blood hemoglobin concentration was maintained in some of the chronic cases with glomerulonephritis but not in nonglomerular renal diseases (118). Uremic patients present with inappropriately low levels of erythropoietin, both in bioactivity (74, 217, 460, 470, 527) and in immunoreactivity (82, 419, 421, 470, 507, 508, 657, 669).

Acute (82) or chronic (469) hypoxic stress may result in greatly increased plasma erythropoietin activity in patients with chronic renal failure. This finding indicates that the capacity to produce erythropoietin is not necessarily abolished in uremia. Instead, the mechanisms that control the synthesis of erythropoietin may operate at a lower set point in some of the patients with chronic renal failure (82). A case has been reported of increased plasma erythropoietin in a uremic patient following switching from hemodialysis to continuous ambulatory peritoneal dialysis with its more effective removal of substances in the middle molecular size (83). On the basis of the observation that interleukin-1 and tumor necrosis factor- α inhibit the *in vitro* production of erythropoietin (see sect. IXB), we proposed that these inflammatory cytokines could suppress the production of erythropoietin at injured renal and extrarenal sites

(317a, 322). There is a rapid decline in the plasma erythropoietin activity during the early phases of renal transplant rejection (541) or acute renal failure of varied etiology (391a, 487).

2. Anemia of chronic disorders

Mild anemia frequently develops in patients with chronic infections, autoimmune diseases, or malignancies. The factors causing this so-called anemia of chronic disorders include insufficient iron availability in the bone marrow, inhibition of the proliferation of erythrocytic progenitors by inflammatory cytokines, increased hemolysis, and bleeding (25, 79, 129, 329, 558, 579, 713). Earlier bioassay data suggested an additional lack of erythropoietin in a variety of inflammatory and malignant diseases (103, 108, 129, 511, 673, 674, 713). Immunological measurements have in general confirmed that plasma erythropoietin levels are inappropriately low for the degree of the anemia in patients with rheumatoid arthritis (24, 286, 557a, 666), AIDS (608), and cancer (439), with few exceptions (45, 584).

Evidence suggesting a modulating role for the immune system on the production of erythropoietin was first provided by the demonstration of elevated plasma erythropoietin levels in hypoxemic mice after thymectomy (418). The authors postulated the release of inhibitors of the formation of erythropoietin by the lymphatic system. Infectious and inflammatory processes induce the production of immunomodulatory peptides by leukocytes, tissue macrophages, fibroblasts, and endothelial cells. In turn, these peptides, or cytokines, are involved in the control of the immune reactions and of hemopoiesis (12, 21, 489). Regarded as important mediators of the anemia of chronic disorders, interleukin-1 (328) and tumor necrosis factor- α (48, 327, 330, 643) have been shown to lower red blood cell production when given to humans (48) or experimental animals (327, 328, 330, 643). These effects have been explained primarily by inhibition of the action of erythropoietin on its targets. The influence of immunomodulatory peptides on the production of erythropoietin still needs to be clarified. Human tumor necrosis factor- α failed to lower the level of circulating erythropoietin in anemic mice (330). Murine interferon- α inhibited the erythropoietin response in hypoxemic mice (291). Interleukin-1 and tumor necrosis factor- α suppressed the *in vitro* synthesis of erythropoietin in cultures of the human hepatoma cell lines HepG2 (317a, 322) and Hep3B (174). Interleukin-6, transforming growth factor- β , or interferon- γ did not lower the production of erythropoietin in HepG2 cells. Importantly, the suppression by interleukin-1 was recently confirmed in isolated perfused rat kidneys (317a).

In conclusion, the role of immunomodulatory peptides in the pathophysiology of the production of erythropoietin is only beginning to be understood. Of note, interleukin-1 and tumor necrosis factor- α are generated in several of those renal and nonrenal diseases that are associated with anomalously low plasma eryth-

ropoietin levels, including glomerulonephritis, renal graft rejection, rheumatoid arthritis, AIDS, and malignancies (322).

X. CLINICAL APPLICATION

A. Replacement Therapy in Renal Failure

The first evidence of the potential of the hormone to stimulate erythropoiesis in the anemia of renal failure was provided with the occurrence of reticulocytosis in uremic patients following the infusion of erythropoietin-rich plasma from humans with aplastic anemia (167). Subsequent studies showed that the administration of erythropoietin alleviated the anemia of chronically uremic rats (661) and sheep (164). In mice suffering from uremia and anemia due to inherited polycystic kidneys, reticulocytosis occurred, and hematocrit values increased above normal within 5 wk following ectopic transplantation of human erythropoietin-producing renal carcinoma tissue (655).

Today's success with recombinant human erythropoietin has fully confirmed Erslev's (150) prediction of 1953 that the availability of the hormone in sufficient amounts should make it possible to correct the anemia of chronic renal failure. Extensive clinical studies have shown recombinant erythropoietin, given intravenously (13, 101, 160, 162, 623, 658, 688) or subcutaneously (50, 290, 604), restores the hematocrit and abrogates the blood transfusion requirement in patients on regular hemodialysis therapy (162, 688; for overview see Refs. 6, 9, 314, 524, 576) or continuous ambulatory peritoneal dialysis (290, 396, 611). The relevant question of the cost effectiveness of expensive recombinant erythropoietin treatment has been discussed in references 256 and 591a.

Because uremic sera contain inhibitors of erythropoiesis (201, 217, 376, 419, 421, 670) that might differ between dialysis and predialysis patients, it was important to show that recombinant human erythropoietin also corrects the anemia in predialysis patients with progressive renal failure (163, 387, 388, 493, 617, 636). Anemia has been considered a hemodynamically favorable adaptation to chronic renal disease in rats (226). However, the correction of the anemia in predialysis patients was shown to have no adverse effects on renal function (387, 388, 493, 636).

Recombinant human erythropoietin raises hematocrit and blood hemoglobin concentration in a dose-dependent and predictable way (6, 162, 387), resulting in increased well-being (168) and exercise tolerance (387, 407, 549) of the patients with chronic renal failure. Freedom from blood transfusions avoids the risks of incompatibility reactions, viral infection, and iron overload. In addition, the lack of transfusion-induced human leukocyte antigen sensitization improves kidney transplant survival rates (258).

The peripheral O_2 supply increases with correction of anemia. Transcutaneously measured O_2 tension more

than doubled in patients with chronic renal failure during erythropoietin replacement therapy (104). Echocardiographic studies have shown a reversal of the anemia-induced hyperdynamic cardiac state, indicated by decreased left atrial diameter, ventricular mass index, ventricular end-diastolic diameter, ejection volume, cardiac index, and ventricular acceleration time (392, 565). Impaired brain function in uremia may also improve with correction of anemia (694), as confirmed by electroencephalograph analysis of evoked potentials (257). Cerebral blood flow has been shown to normalize with increasing hematocrit (331).

Uremic subjects with hematocrit values <0.30 (hemoglobin <100 g/l) have been considered eligible for erythropoietin replacement therapy (9, 161) provided they suffer from severe symptoms of anemia (464). The dosage schedules are still a matter of debate. Recommendations for the initial dose vary between three times per week, 150 U/kg body wt in some countries (9, 161) and 40 U/kg body wt in others (464, 623). The doses are usually lowered in the long term to maintain hematocrit at ~ 0.35 (9, 161). A further increase in hematocrit is not desirable because of the risks of development of hypertension and thrombosis. The target hematocrit is usually reached after 1–2 mo. Reasons for poor responses include iron deficiency, aluminium overload, active inflammatory disease, and osteitis fibrosa. Iron supplementation is usually required in patients not previously iron overloaded (9, 161). On the other hand, transfusion-induced hemosiderosis may be cured by stimulation of erythropoiesis with exogenous erythropoietin in combination with repeated bloodletting (413). By raising hematocrit, erythropoietin improves the defective hemostasis in uremic patients. The prolonged bleeding time is shortened (243, 331, 458, 660). As yet, there has been no case reported of the formation of antibodies to recombinant human erythropoietin in humans.

Influenza-like symptoms occur occasionally when recombinant proteins purified from tissue culture media are given intravenously. As first noted by Winearls et al. (688), some patients report feelings of cold or warmth and episodes of abdominal cramps or myalgias after the injection of recombinant erythropoietin. Although these symptoms are transient and do not limit therapy, it would appear important to identify the nature of the contaminants responsible for it.

Development or worsening of hypertension is generally considered the most serious adverse effect of the treatment of the anemia of renal failure with erythropoietin (530). Hypertensive encephalopathy and seizures have been reported. Statistical analyses indicate, however, that the number of seizure episodes is not different from the incidence in uremic patients not treated with erythropoietin (160, 161). The prevalence of hypertension is 15–35% (13, 160, 161, 623). Its frequency appears to be higher in patients with a previous history of hypertension (80). Clearly, the baroreceptor-mediated regulation of the arterial blood pressure must be impaired in the uremic patients who develop hypertension. In contrast with selected case reports (61, 482), a recent

study in 100 each of hemodialyzed and peritoneal dialyzed patients failed to demonstrate elevated blood pressure when hematocrit was significantly increased by blood transfusion and removal of uremic toxins (687).

The increase in mean arterial blood pressure primarily results from increased peripheral resistance due to the elevated blood viscosity and the loss of hypoxia-induced vasodilation (331, 493, 524, 530, 531). The idea that the increased hemoglobin in circulation may cause vasoconstriction by trapping nitric oxide (400) appears less likely because nitric oxide is completely bound when the hemoglobin concentration in blood is as low as 40 g/l (659). Aldosterone and renin activity are not elevated in plasma during erythropoietin therapy (304, 352, 662). Activation of the sympathetic nervous system has been suggested (304), but other investigators did not measure increased norepinephrine levels (662). With respect to stress exposure, it is of note here that erythropoietin-treated hemodialyzed patients have no increased participation in paid employment and in social activities (694). A direct acute pressor action of recombinant erythropoietin is unlikely, as blood pressure does not usually increase immediately after the injection of the drug. Neither does hypertension occur when subjects without renal disease are treated with erythropoietin (9). It has also been shown that recombinant erythropoietin in the clinically used concentrations does not increase in vitro vascular resistance in the isolated perfused rat kidney (500) and in human subcutis preparations (66). In extremely high concentration ($>10,000$ U/l), not usually reached in vivo, commercial preparations of recombinant human erythropoietin caused constriction of isolated renal and mesenteric resistance vessels (281). However, whether this vasoconstriction was due to intrinsic erythropoietin activity has remained unproven, because receptor binding studies and control experiments with the vehicle and with antierythropoietin antibody were missing. Erythropoietin ampules for clinical use contain additives such as human serum albumin or Tween 20 for stabilization.

B. Pharmacological Use in Other Anemias

The benefits of the prescription of recombinant human erythropoietin have been investigated recently in the anemias of other, nonrenal chronic disorders, including autoimmune diseases, malignancies, and AIDS. High doses of erythropoietin were shown to correct the anemia of rheumatoid arthritis (427, 520, 630). Similarly successful were trials in anemic patients with multiple myeloma or non-Hodgkin's lymphoma (395, 495). Initial evidence has been provided for the efficacy of erythropoietin in the treatment of chemotherapy-induced anemia (494). Importantly, in vitro studies have shown that erythropoietin does not affect the growth of malignant human cells (33a). In AIDS patients, whose anemia may be aggravated by zidovudine treatment, the application of erythropoietin reduced the need for blood transfusion if the endogenous plasma erythropoietin concentration was <500 U/l (183). As noted (321, 326), the demonstra-

tion of an inappropriately low level of circulating erythropoietin is in general a prerequisite for the beneficial application of the recombinant product. In various models of anemia in rodents, repeated injections of erythropoietin failed to accelerate the recovery from anemia unless the endogenous production of erythropoietin was impaired (105, 403) or extremely high doses of the recombinant product were given (405). In a clinical trial, the intravenous application of erythropoietin resulted in an increase of the blood hemoglobin concentration in 3 of 12 patients with myelodysplastic syndromes or aplastic anemia despite high endogenous levels of the hormone (41). Subcutaneous administration was ineffective in this case (660a). Possibly, the less erythropoietin-sensitive immature erythrocytic progenitors and stem cells (see sect. VIII.4) are triggered into proliferation when the blood level of erythropoietin is extremely elevated by intravenous application of the drug in escalating doses.

Anemia of prematurity develops in babies born before week 32 of gestation at 4–6 wk after birth. Recombinant human erythropoietin was shown to stimulate the *in vitro* growth of erythrocytic progenitors from cord blood of neonates (542). Although the use of the recombinant product makes it possible to increase hematocrit in premature infants (272), the benefit of this treatment is not clear, in part because the main clinical problems of prematurity are independent of the severity of anemia. There is a lack of a clear relationship between hematocrit and either heart and respiratory rate or the incidence of apnea and bradycardia (345, 614). However, if hematocrit is to be raised as a precaution against poor growth of the neonate (62), the administration of erythropoietin should prove to be preferable to the transfusion of erythrocytes (591).

Understandably, an increasing number of patients refuse blood transfusions, since an alternative has become available. Recombinant human erythropoietin is presently being investigated as a treatment for many types of anemia. It is effective in the maternal anemia postpartum (111). It may prove useful for stimulation of erythropoiesis in certain rare disorders, such as reported in Gaucher's disease (546). However, some hopes may not meet with success. For example, erythropoietin therapy failed to reduce the rate of hemolysis by enhancing hemoglobin F levels in patients with sickle cell anemia (233).

In elective surgical procedures, the transfusion of preoperatively collected autologous blood eliminates the infectious and immunological hazards of donor blood (338). Despite the development of anemia, the level of endogenous erythropoietin increases only slightly in response to repeated phlebotomy in autologous blood donors (346). Trials in baboons first showed that the use of erythropoietin can increase the yield of blood units by 35% in the course of an aggressive phlebotomy program (384). A multicenter study in humans has confirmed recently the ability of erythropoietin treatment to facilitate autologous blood donation (242). Aside from the possibility of improving autologous blood donation, the severity and the duration of postoperative anemia might

be lessened by the application of recombinant human erythropoietin some days before surgery (383a).

XI. COMMENTS

The availability of recombinant humanlike erythropoietin is a major advance in clinical medicine. Replacing the native hormone, the recombinant product restores the concentration of red blood cells in patients suffering from chronic renal failure. In addition, there are encouraging reports of its potency in alleviating the anemia of chronic inflammatory and malignant diseases and in enhancing erythropoiesis in surgical patients, thus reducing their need for homologous blood transfusion.

The chemical structure of recombinant human erythropoietin has been elucidated in detail. The polypeptide chain of 165 amino acids has one site with *O*-linked and three sites with *N*-linked oligosaccharides, which are required for full biological activity *in vivo*. One reason for the lack of major side effects of the therapy with recombinant human erythropoietin is its identity with the natural hormone. Another is the restricted action of erythropoietin.

Erythropoiesis extends from the small pool of pluripotent myeloid stem cells to the greatly amplified number of reticulocytes. In the growth pyramid of erythrocytic tissue, erythropoietin primarily stimulates the proliferation and differentiation of the committed progenitors, BFU-E and CFU-E. Commitment to the erythrocytic lineage is associated with the expression of specific erythropoietin receptors on the cell membrane. The genes encoding the human and the murine erythropoietin receptor have been isolated. Studies of the structure and expression of the erythropoietin receptor may provide new insights into the pathogenesis of hemopoietic disorders such as aplastic anemia or polycythemia vera. In addition, the structural similarity with the receptors of other hemopoietic growth factors is of evolutionary interest. Following receptor binding of erythropoietin, the ligand-receptor complex undergoes endocytosis. Apparently mediated through activation of a GTP-binding protein, erythropoietin induces a rapid increase in intracellular free calcium concentration in erythrocytic progenitors. The subsequent mechanism of signal transduction to the nucleus still needs to be explored.

Although erythropoietin has become an important pharmacological agent, major questions of the physiology of its production have remained unanswered. There are conflicting reports on the cellular origin of the hormone in the kidney and the liver. According to the prevailing evidence, erythropoietin is synthesized by either peritubular capillary or tubular cells in the kidney and by epithelial or Kupffer cells in the liver.

Tissue hypoxia is the main stimulus for erythropoietin gene transcription. The level of erythropoietin mRNA has been shown to increase greatly in the kidneys and the liver of anemic or hypoxemic rodents. The relevant mechanism of O₂ sensing is still poorly understood. A renal cell system for the *in vitro* investigation

of the production of erythropoietin is not available. Studies with erythropoietin-producing hepatoma cell cultures have provided some evidence to propose that the O₂ sensor in control of the synthesis of erythropoietin is a heme protein, possibly involving microsomal MFOs. In addition, evidence has been provided to assume that protein kinases A and C are involved in the elaboration of erythropoietin.

Molecular biology studies have progressed from the isolation and characterization of the human erythropoietin gene to the analysis of its expression in transgenic mice. Apparently different DNA sequences control erythropoietin production in the kidney and the liver. The generation of transgenic mice with other gene constructs may enable investigators to identify the relevant *cis*-regulatory elements and *trans*-acting factors, thus leading to a better understanding of the link between O₂ deficiency and activation of the erythropoietin gene.

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See *Estrogen Receptor*

Erythropoietin, Biochemistry of

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- I. INTRODUCTION
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Erythropoietin (EPO) is a cytokine that stimulates erythropoiesis, the formation of red blood cells. The mature EPO protein consists of 166 amino acids; its predicted tertiary structure is an anti-parallel bundle of four α -helices. The primary site of EPO production is the kidney. In addition to tissue-specific control, regulation of EPO production occurs mainly at the transcriptional level, and the production of EPO can be induced under anemic and hypoxic conditions.

I. INTRODUCTION

Oxygen is essential for life. The oxygen consumption of the human body varies based on an individual's metabolic demand and can be limited by the environmental oxygen concentration. Early observations provided links between symptoms such as fatigue, shortness of breath, and cardiac palpitations and either low oxygen tension or anemia. Further studies showed that low oxygen tension resulted in an increased number of red blood cells, the cells responsible for the delivery of oxygen to systemic organs and tissues.

The red blood cell was the first identified blood cell type, and its oxygen-carrying function by hemoglobin was observed by Hoppe-Seyler in the 19th century. However, the regulation of red blood cell formation, or erythropoiesis, remained unknown until 1906 when Carnot and Deflandre first introduced the idea of a humoral mediator for erythropoiesis. Almost 50 years later, Erslev and Reissmann formally tested this hypothesis experimentally by injecting serum from anemic rabbits into normal rabbits and noting a significant increase of red blood cells in the recipient animals. They further proved this hypothesis by using a parabiotic model in rats to demonstrate a circulating erythropoiesis-stimulating factor, now known as erythropoietin (EPO).

II. BIOCHEMICAL PROPERTIES OF EPO

Biochemical characterization of EPO became possible only after Goldwasser and colleagues obtained

partially purified protein from the urine of anemic patients in 1977. The molecular characterization of EPO was further advanced when Lin *et al.* and Jacobs *et al.* cloned the cDNA encoding human EPO in 1985. The human protein consists of a 193-amino-acid sequence with a 27-residue signal leader sequence, resulting in a 166-amino-acid mature protein (Fig. 1). The protein contains two disulfide linkages and multiple glycosylation sites. Sequence analysis indicates that EPO belongs to a family of cytokines, including growth hormone, prolactin, colony-stimulating factor, and the interleukins. The predicted EPO tertiary structure is an anti-parallel bundle of four α -helices, structurally analogous to the growth hormone molecule. The molecular weight of mature EPO is approximately 30.4 kDa with 40% of its mass derived from carbohydrates. As shown in Fig. 1, the mature protein contains one O-linked glycosylation site (Ser-126) and three N-linked glycosylation sites (Asn-24, Asn-38, and Asn-83). Different carbohydrate structures can be found on the same glycosylation site: e.g., the N-linked chains may

contain two, three, or four branches, each ending with a sialic acid molecule. The O-linked site may contain zero to two sialic acid molecules. This posttranslational modification gives rise to heterogeneous EPO molecules with different numbers of sialic acid molecules attached, thereby varying the net negative charge. In order to study the effects of this microheterogeneity on EPO's biological activity, different isoforms of EPO were isolated and injected into animals. Isoforms with higher sialic acid content have greater *in vivo* biological activity and a longer serum half-life. In contrast, higher sialic acid content leads to lower *in vitro* receptor-binding affinity. These results suggest that the serum half-life and clearance rate have stronger effects on EPO's biological activity *in vivo* than does receptor-binding affinity.

III. EPO LEVELS AND ITS REGULATION

Although hypoxia clearly stimulates EPO production, how and where this is accomplished was unclear until Jacobsen demonstrated that nephrectomized animals

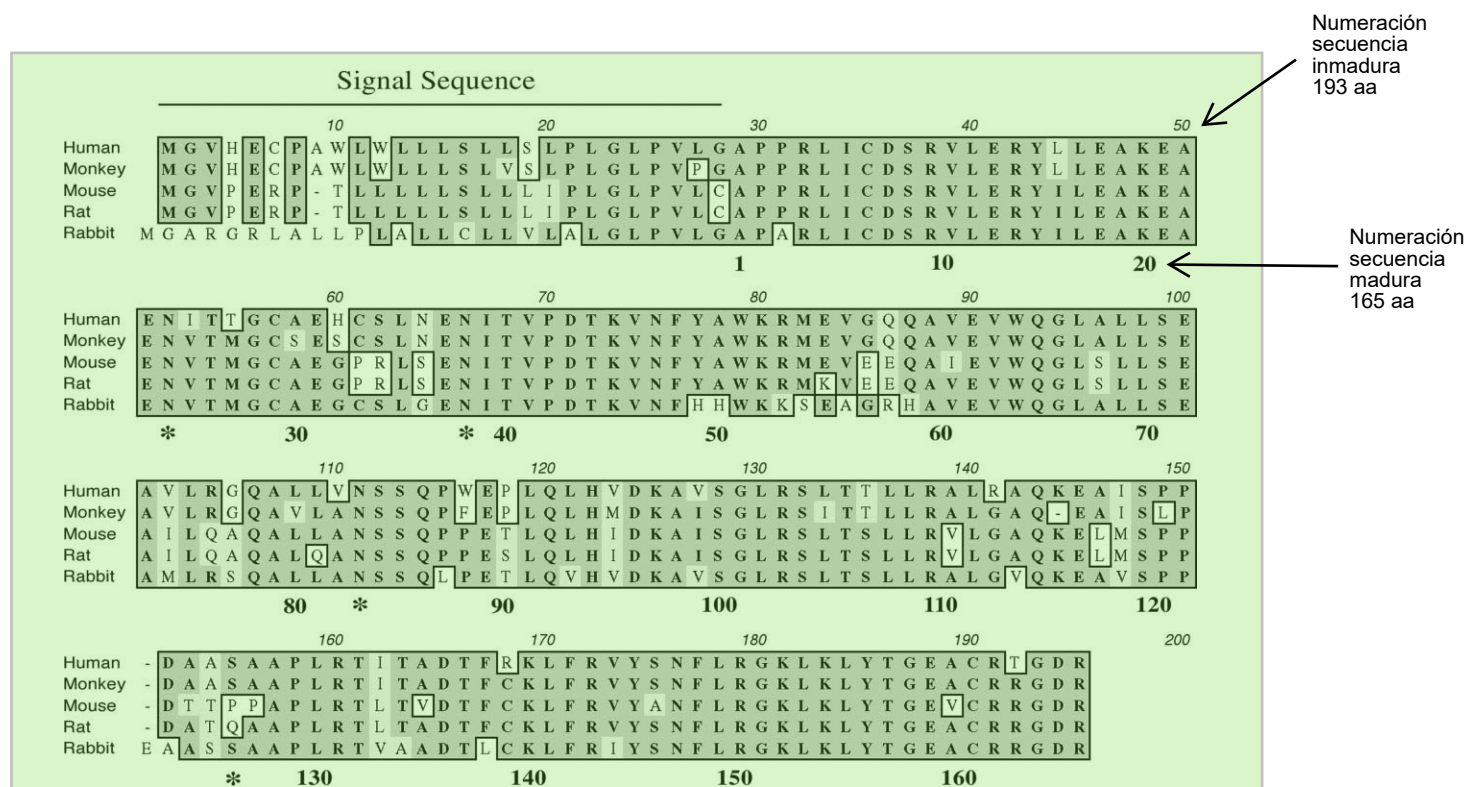


FIGURE 1 Alignment of the EPO amino acid sequence across human, monkey, mouse, rabbit, and rat showing a high degree of homology. Signal sequence comprises approximately the first 27 residues depending on the species. Glycosylation sites are marked with an asterisk below the asparagine (N) or serine (S) residues in relation to the human sequence. Boldface numbers below the alignment represent numbering of the mature sequence, and numbers at the top of the alignment represent numbering from the start of the signal sequence. Dark shaded residues indicate identity and lightly shaded residues indicate similarity.

failed to mount an EPO response to hypoxia, suggesting that the kidney was the primary site of EPO production in adult animals. Koury and Lacombe in 1988 localized erythropoietin expression to interstitial cells close to the proximal tubular cells of the kidney by *in situ* hybridization. The kidney is the primary site for EPO production, at least in part due to its function in sensing oxygen tension and extracellular volume. By regulating red cell mass through EPO production and plasma volume through excretion of salt and water, the kidney sets the hematocrit at a normal value of 45%. The adult liver is also capable of producing approximately 10–15% of the circulating EPO in anemic rats. These levels, however, are not sufficient to sustain a normal level of erythropoiesis in the absence of renal EPO production.

EPO released into the blood circulation travels to major erythropoietic organs, such as the bone marrow and the spleen (in rodent), and binds to its cell surface receptor (EPOR) on erythroid-specific precursors to stimulate red blood cell production and increase the peripheral red cell mass. In the normal healthy individual, the serum EPO level is very low, ranging from 10 to 30 mU/ml. No sex-related differences in EPO levels have been observed and EPO levels are not affected by the menstrual cycle.

In addition to tissue-specific control, regulation of EPO production occurs mainly at the level of transcription and can be induced under anemic and hypoxic conditions. *EPO* mRNA levels increase 200- to 1000-fold in mice with bleeding-induced anemia or severe hypoxia in order to compensate for the blood loss and reduced oxygen-carrying capacity. *EPO* expression is also carefully regulated to prevent the overproduction of red blood cells. In anemic mice, the level of EPO induction is proportional to the level of anemia induced. Abnormally high red cell mass or hematocrit, clinically defined as erythrocytosis, can increase blood viscosity and cause hypertension. A considerable amount of research has been invested in determining how the body is able to sense the changes in oxygen tension and how that translates into EPO production. Interestingly, EPO is found only in placental mammals. An 80–82% amino acid identity exists between the human sequence and that of monkey, mouse, rat, and rabbit (Fig. 1). Studies on the *EPO* locus itself have uncovered several transcriptional regulatory elements. The *EPO* gene consists of five exons and four introns. In addition to a high level of conservation within the *EPO* coding sequence across mammalian species, regions in the promoter, the first intron, and a small region 3' to the

polyadenylation site are also highly conserved. These similarities suggest the existence of conserved transcriptional regulatory mechanisms. Studies using transgenic mice carrying different regions of the putative regulatory sequences demonstrated that (1) *cis*-acting elements for tissue-specific and developmentally regulated *EPO* expression lie outside the 400 and 700 bp of 5'- and 3'-flanking sequences, respectively, and (2) *cis*-acting sequences within the 400-bp 5' and 700 bp 3'-flanking regions are capable of mediating an oxygen-related response. Subsequent studies with larger regions of the 5'-promoter regions identified a region 0.4 to 6 kb upstream of the *EPO* gene that may possess binding sites for functionally relevant repressors. Further upstream, kidney-specific inducible elements were found between 9.5 and 14 kb upstream of the *EPO* gene. Study of the conserved region 3' to the polyadenylation site (Fig. 2) also provided information crucial for *EPO* regulation. A hypoxia-inducible enhancer was discovered in a region 120 bp 3' to the polyadenylation site. Its activity is independent of orientation or distance from the promoter. Fine-mapping of the 3'-enhancer sequence reveals three sites critical for regulation of the *EPO* gene by hypoxia (Fig. 2). The first site on the 5'-end has the sequence TACGTGCT, which is the consensus binding site for the transcription factor hypoxia-inducible factor-1a (HIF-1a) and the aryl hydrocarbon receptor nuclear translocator (ARNT) heterodimeric complex. This binding site is essential for HIF-1 dependent and hypoxia-induced *EPO* expression. A second site lies only 7 bp 3' of the HIF-1 binding site with the sequence CACA. Although no protein is known to bind to this sequence, mutating this site leads to loss of the hypoxia-inducible function of the enhancer. The third site is located 10 bp further downstream from the CACA site and consists of two consensus binding sites for steroid hormone receptor. Screening for nuclear hormone receptors capable of binding to this site led to the identification of HNF-4. Using Hep3B, a liver cell line capable of producing EPO in response to hypoxia, HNF-4 is shown to be required for the response to low oxygen levels. Introducing dominant negative HNF-4 into Hep3B cells abolished their ability to respond to hypoxia by inducing *EPO* expression. Moreover, like *EPO*, HNF-4 is also expressed in the renal cortex of the kidney and the liver.

The aforementioned *cis*-acting element in the 5'-promoter region also has a role in the response to hypoxia, maintaining a 10-fold induction in response to hypoxia in the absence of the 3'-enhancer. In the

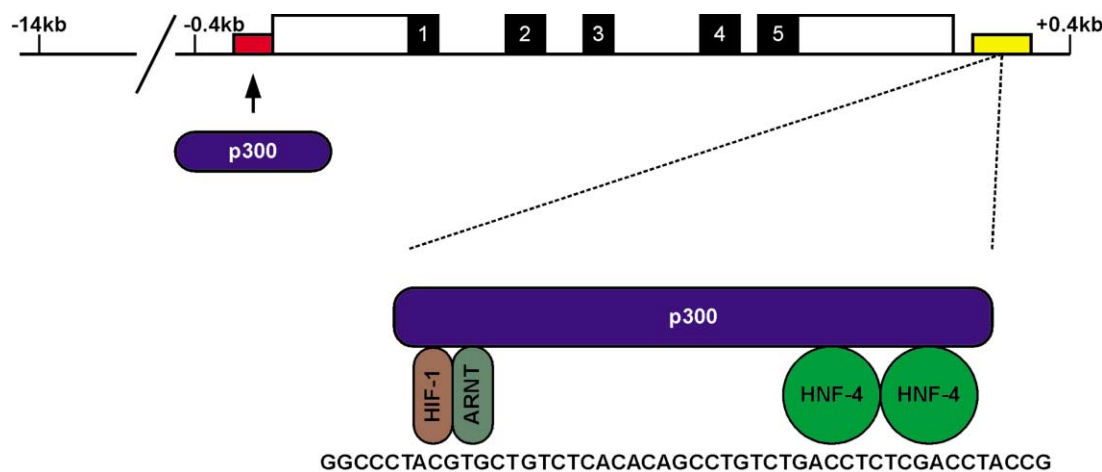


FIGURE 2 *EPO* gene regulation in adults. Coding regions of the five exons are shown in black. The 5'- and 3'-untranslated regions are shown as boxes. The 3'-enhancer (small box in upper right-hand side) is expanded. p300 is capable of binding and activating the minimal promoter (small box in upper left-hand side) and can act synergistically with the 3'-enhancer to increase *EPO* transcription in response to hypoxia. Consensus DNA sequences for hypoxia-inducible expression are underlined. Adapted from Ebert, B. L., and Bunn, H. F. Regulation of the erythropoietin gene. *Blood* 1999; 94: 1864–1877. Copyright American Society of Hematology. Used with permission.

presence of the 3'-enhancer, the 5' *cis*-acting element synergistically activates *EPO* expression by 40-fold in response to hypoxia. Taken together, the 5'-promoter and 3'-enhancer of the *EPO* gene constitute a potent transcriptional control unit, which regulates *EPO* production in response to hypoxic conditions or anemic states.

IV. EPO AND EPOR: SIGNAL TRANSDUCTION

Given the thoroughness of our understanding of *EPO* signaling and the structural and functional similarities between members of the cytokine receptor superfamily, *EPO* signaling can serve as a model for understanding cytokine signal transduction in general. *EPO* signals through its cognate receptor, the EPOR, a protein expressed on the surface of appropriate target cells. Because the EPOR lacks any known catalytic function, it relies on the activity of associated Janus kinase 2 (JAK2). Upon *EPO* binding to dimerized EPORs, JAK2 molecules are brought into close proximity, phosphorylating and thereby activating one another. Activated JAK2 then phosphorylates individual tyrosine residues within the cytosolic region of EPOR, thereby creating docking sites for recruiting SH2 domain-containing signaling molecules, including (1) signal transducer and activator of transcription 5 (STAT5), molecules of which, once phosphorylated, dimerize and translocate into the nucleus, where they initiate transcription of target genes; and (2) the regulatory subunit of

phosphatidylinositol 3-kinase (PI3K), which activates other downstream signaling molecules, such as serine/threonine kinase Akt; and (3) Grb2, an adapter molecule that mediates activation of the Ras-mitogen-activated protein kinase (MAPK) signaling pathway (Fig. 3). Ultimately, these and other signaling cascades produce alterations in the patterns of gene expression. Such "target genes" in erythrocytes include those involved in cellular proliferation (e.g., *c-myc* and *c-fos*) and survival (e.g., *Bcl-X_L*), as well as cell type-specific genes, such as those encoding globins (the protein component of hemoglobin), the transferrin receptor (which mediates entry of iron into cells), and certain membrane structural proteins (which contribute to the characteristic biconcave structure of erythrocytes).

Although it has long been presumed that cytokine signaling must be negatively regulated with respect to both duration and intensity, only in recent years has a better understanding of this process been achieved. To generate more precise control over cytokine signaling, several mechanisms of negative regulation exist. First of all, the hematopoietic protein tyrosine phosphatase SH-PTP1 (also called HCP and PTP1C) associates through its SH2 domain with a specific phosphotyrosine residue of the EPOR and is responsible for dephosphorylating and thus inactivating JAK2. In fact, mutating this tyrosine to phenylalanine renders cells that express the mutant receptor hyperresponsive to *EPO* stimulation.

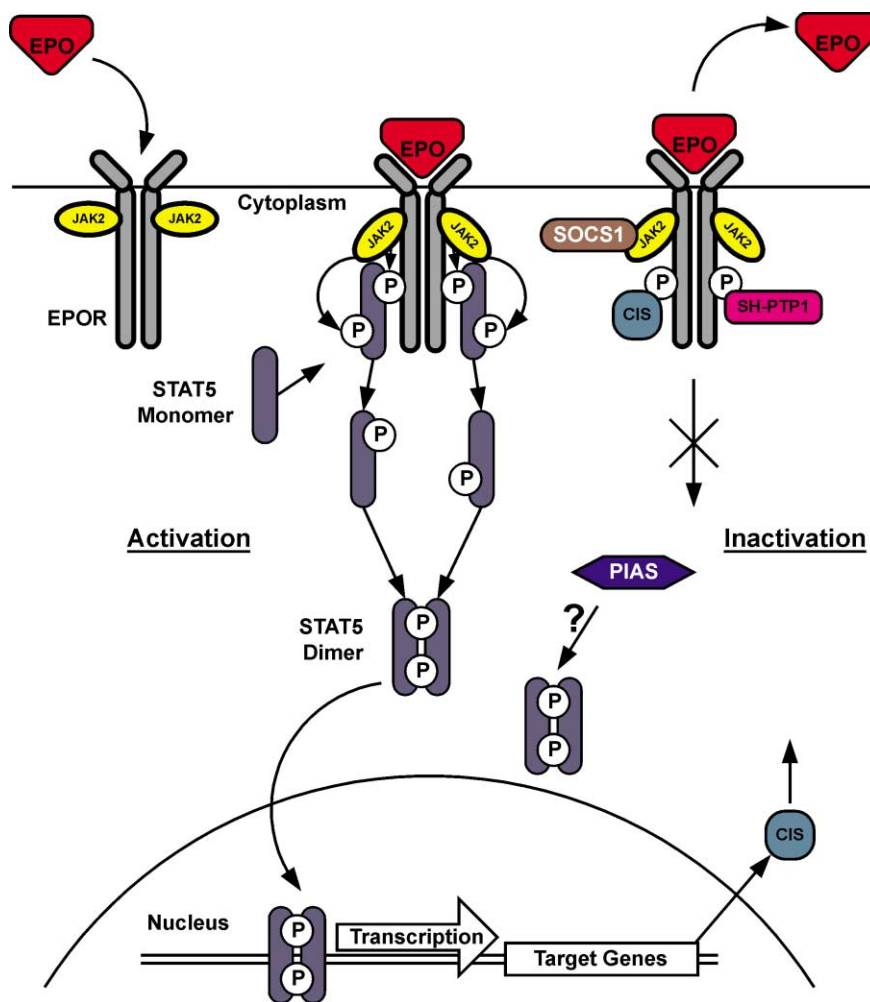


FIGURE 3 EPO/EPOR signal transduction. Activation: EPO binds the EPOR and activates JAK2. JAK2 then phosphorylates the tyrosine residues of the EPOR cytoplasmic tail, which provides a docking site for recruiting the SH2 domain-containing signaling molecules, such as STAT5. STAT5 is subsequently tyrosine phosphorylated, dimerizes, and translocates to the nucleus to activate the transcription of target genes. Inactivation of the receptor occurs through various means, including binding of the receptor and JAK2 molecule by various inhibitors, such as CIS and SOCS1, and dephosphorylation of JAK2 by SH-PTP1 phosphatase. It is unclear whether PIAS molecules participate in negative regulation of EPOR signaling by modulating the function of STAT5.

Naturally occurring variants of the human *EPOR* lacking the C-terminal negative regulatory domain, including this tyrosine residue, may give rise to familial erythrocytosis.

Also contributing to the attenuation of cytokine signaling are the protein families PIAS (protein inhibitors of activated STATs, with four mammalian family members identified) and SOCS (suppressor of cytokine signaling, with eight known mammalian family members). Although the role of PIAS family members in EPO signaling has not been addressed, multiple SOCS family members appear to counteract

EPO signaling by acting on different targets. Whereas SOCS1 appears to bind to JAK2 to inhibit its activity, CIS, another SOCS family member, binds to the EPOR itself (1) to compete with STAT5 for binding to specific phosphotyrosine residues and (2) possibly to deliver active signaling complexes to the proteasome for degradation.

Finally, EPO signaling may be modulated by cell surface expression of the EPOR and EPO-EPOR complex internalization and degradation. The mechanisms controlling these events are poorly understood.

V. ANIMAL MODELS FOR EPO FUNCTION

Whereas biochemical analysis has helped identify key players involved in EPO signaling, genetic analysis, involving either the fruit fly, *Drosophila melanogaster*, or the house mouse, *Mus musculus*, has provided insight into their functional relevance. The hemolymph of insects is analogous to mammalian blood, in that it carries nutrients and cells involved in immunity. *D. melanogaster* does not need red blood cells as an oxygen carrier and sequencing information derived from the fly genome project shows no homologue to either EPO or EPOR. Nevertheless, signaling molecules known to be important for EPOR signaling do exist in the fly. In fact, gain-of-function mutations of the JAK homologue *hopscotch* lead to the overproliferation and premature differentiation of larval blood cells.

Although *D. melanogaster* permits powerful genetic analysis due to its low maintenance cost and short generation time, the mouse is physiologically more relevant to humans. A powerful technique for studying the role of a particular gene in mice is achieved through homologous recombination in embryonic stem cells. Mice lacking EPO or EPOR die with severe anemia at approximately embryonic day 13 (mouse gestation lasts for 18–21 days,

depending on strain, diet, and other factors). Interestingly, EPO and EPOR are unnecessary for erythroid commitment, but instead are required for their terminal differentiation into mature red blood cells. Thus, as shown in Fig. 4, whereas normal fetal livers contain cells at various differentiation stages within the erythroid lineage, livers lacking EPO or EPOR are completely pale and fail to produce mature erythrocytes. Furthermore, “primitive” erythropoiesis, which occurs very early in development in the yolk sac compartment, appears to be intact in either knockout. This result would suggest that EPO and EPOR are necessary only for the production of adult-type or “definitive” red blood cells, which are generated at midgestation throughout adulthood. Finally, the fact that both deficient mouse strains exhibit very similar, if not identical, phenotypes suggests that only one receptor exists for EPO and that no other naturally occurring ligands or receptors can replace either protein.

JAK2-deficient mice are very reminiscent of EPO- and EPOR-deficient mice as they too die as embryos with anemia. However, the degree of anemia is greater in JAK2 null mice, which is not surprising since JAK2 is implicated in signaling by many cytokines other than EPO, including thrombopoietin, interleukin-3, and granulocyte/macrophage-colony-stimulating

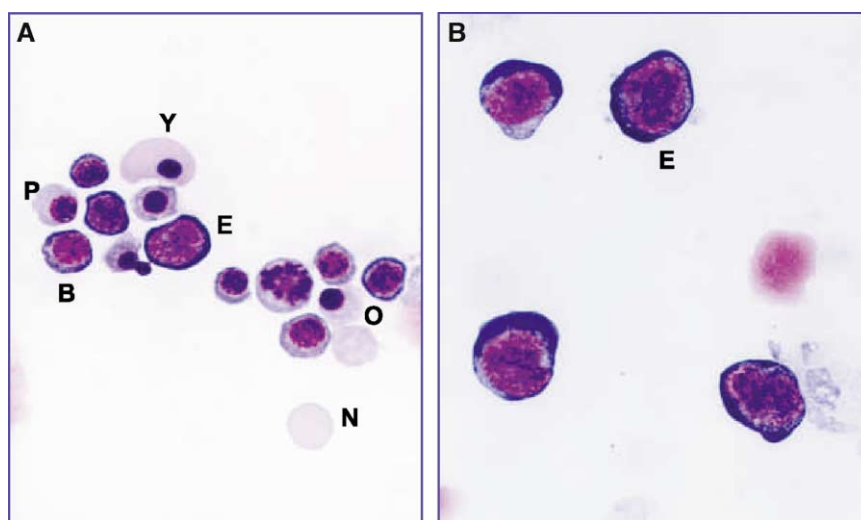


FIGURE 4 EPO is essential for the differentiation of erythroid progenitor cells in the fetal liver. Cytopsin preparation of liver from a wild-type (A) and a homozygous mutant (B) liver stained with Giemsa. Numerous erythropoietic cells at all stages of differentiation are seen in the WT fetal liver, including proerythroblasts (E), basophilic erythroblasts (P), orthochromatic erythroblasts (O), and nonnucleated erythrocytes (N). Yolk sac-derived nucleated erythrocytes (Y) are also seen. In the mutant liver preparation, proerythroblasts are prominent with a noticeable absence of cells at later stages of differentiation. Reprinted from *Cell* 83, Wu, H., Liu, X., Jaenisch, R. and Losish, H. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. 56–67, 1995. With permission from Elsevier Science.

factor, all of which can positively regulate erythropoiesis although they are dispensable for steady-state erythropoiesis. Thus, *JAK2*, like *EPO* and *EPOR*, is both a critical and nonredundant player in erythropoiesis.

Among seven mammalian STAT family members, STAT5A and STAT5B have been implicated to be involved in EPO signaling based on *in vitro* experiments. However, disrupting either or both genes in mice does not result in erythropoietic defects similar to that in *EPO*, *EPOR*, or *JAK2* mutant mice, although deficiencies exist in signaling by other cytokines, namely, prolactin and growth hormone. Possible explanations for the relatively subtle effects of STAT5 on erythropoiesis *in vivo* include redundancy with other components of EPO signaling that are downstream of JAK2 (e.g., MAPK or PI3K) or possibly the existence of a hitherto unidentified STAT or STAT-related protein that plays a role in EPO signaling *in vivo*.

Members of the SOCS family have also been deleted in mice. Disruption of *SOCS3* results in erythrocytosis so severe that mice die during embryogenesis. In contrast, *SOCS3* overexpression results in a severe anemia, much like what is seen in the *EPO*-, *EPOR*-, or *JAK2*-deficient mice.

VI. CLINICAL APPLICATIONS

The pharmacological use of recombinant human EPO has been well established clinically in treating anemia associated with a variety of pathophysiological conditions. Anemia can be classified by hemoglobin (Hb) levels and subdivided into the following categories: mild (10–12 g/dl), moderate (8–10 g/dl), severe (6.5–7.9 g/dl), and life-threatening (<6.5 g/dl). Hb is contained mostly within erythrocytes, which function in binding, transporting, and delivering oxygen to the periphery. Loss of red blood cells results in a compensatory response by the body to maintain oxygen delivery to tissues. One such response is the induction of *EPO* expression, which subsequently results in an increase in red cell mass.

Anemia can present with a wide spectrum of symptoms involving virtually every organ system, including heart palpitations, pale and cold skin, dyspnea, headaches, dizziness, vertigo, decline in cognitive function, retinal damage, angina, heart failure, left ventricular hypertrophy, proteinuria, decreased libido, impotence, amenorrhea, irregular bowel movement, indigestion, malabsorption, tachypnea, pulmonary edema, generalized edema, and renal failure. Because of their many detrimental

effects on quality of life, anemias can pose significant health problems.

Although they can result from a variety of causes, a large number of anemias in the clinical setting result from a decreased production of red cells. The most promising method in treating this type of anemia has been recombinant EPO. Recombinant human EPO (or Epoetin α) has been approved by the Food and Drug Administration for treatment of anemia associated with chronic renal failure. EPO has also proved to be beneficial for treatment of anemia associated with Zidovudine therapy for patients infected with human immunodeficiency virus, anemia associated with chemotherapy in cancer patients, and anemia associated with surgery. Recent studies have also suggested potential therapeutic uses for EPO in the treatment of disorders such as myelodysplastic syndrome, anemias of prematurity, and congestive heart failure.

Because of its relative safety and efficacy, athletes attempting to improve their oxygen-carrying capacity have frequently abused recombinant EPO. Given their similarity, recombinant EPO cannot be distinguished from endogenous EPO by currently available immunoassays. However, an electrophoresis method can distinguish endogenous EPO and synthetic EPO because of subtle discrepancies in posttranslational modifications that introduce charge differences between the EPO isoforms. In fact, when this technique was employed, urine samples of 28 of 102 tested cyclists of the 1998 Tour de France were found to have electrophoresis patterns indicative of recombinant EPO.

VII. EPO FUNCTION IN OTHER LINEAGES

The role of erythropoietin in stimulating red blood cell formation has been clearly defined. However, recent evidence suggests that EPO may have more diverse roles as well. For instance, *EPOR* expression has been detected in human umbilical cord and placental endothelial cell lines, and EPO was capable of stimulating endothelial cell proliferation *in vitro*. In addition, EPO is known to induce a pro-angiogenic phenotype in cultured endothelial cells and stimulated neovascularization in the chick chorioallantoic membrane. EPO also plays an important role in myoblast proliferation as well as in cardiac morphogenesis.

EPOR expression has also been found in PC12 cells (a cell line with neuronal-like properties), hippocampal and cortical neurons, and astrocytes. EPO-binding sites can be localized in both murine

and human fetal brains. Similar to EPO production in the kidney, endogenous EPO expression in neurons and astrocytes is controlled by oxygen-sensing mechanisms that involve the redox state of the brain. In animal models, EPO has been shown to protect neurons from glutamate-induced as well as ischemia-induced apoptotic cell death. Thus, research toward understanding all the *in vivo* functions of EPO can lead to new and improved therapeutic regimens and indications beyond the treatment of anemias.

VIII. SUMMARY

The study of EPO has gone from the concept that a hormone-like molecule exists and is capable of stimulating red blood cell formation to the cloning of the gene and its biochemical and molecular characterizations. Through decades of study on EPO, research that focused on its biological properties has led to the development of a highly successful therapeutic drug that has significantly improved the quality of human life. Further studies with elegant genetic animal models have proven its *in vivo* biological function, and the dissection of its signaling pathway has progressed to the level of not just exploring what EPO turns on but also what turns off the signal from the EPOR. In an attempt to improve on Mother Nature, researchers have begun to isolate small peptides or to identify small chemical molecules that can mimic EPO function by activating the EPOR. Even though an enormous amount of information has been generated, more remains to be learned, and researchers will strive to better understand the functions of this essential hormone in regulating red blood cell formation and its role in other lineages.

Glossary

- anemia** A significant reduction in red cell mass and a corresponding decrease in the oxygen-carrying capacity of the blood.
- cytokine** A member of a large family of secreted proteins, defined by structural similarities in their receptors, which regulate fundamental biological processes, such as hematopoiesis (the formation of blood cells), immunity, and wound healing.
- enhancer** A *cis*-acting sequence that increases the utilization of eukaryotic promoters and can function in either orientation and in any location (either upstream or downstream), relative to the promoter.
- erythrocyte** A mature red blood cell that circulates within the body to deliver oxygen to tissues and, in exchange, remove carbon dioxide and hydrogen ion from tissues.

erythrocytosis An abnormal increase in red blood cells in the circulating blood. This elevation is usually accompanied by corresponding increases in the quantity of hemoglobin and in the hematocrit.

glycosylation The conjugation of carbohydrate residues, which for proteins can occur through the side-chain oxygen atom of serine or threonine residues by O-glycosidic linkages or to the side-chain nitrogen of asparagine residues by N-glycosidic linkages.

hypoxia A condition in which oxygen tension is lower than the normal level.

kinase Any protein that catalyzes phosphorylation (the transfer of a phosphate group from a donor to a substrate molecule).

See Also the Following Articles

Angiogenesis • *Drosophila* Neuropeptides • Flt3 Ligand
• Protein Kinases

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Erythropoietin, Genetics of

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- I. HISTORY OF ERYTHROPOIETIN
- II. THE ERYTHROPOIETIN GENE
- III. THE ERYTHROPOIETIN PROTEIN
- IV. SITE OF ORIGIN
- V. ERYTHROPOIETIN GENE EXPRESSION
- VI. CLINICAL APPLICATION

Erythropoietin is both a hormone and a growth factor. It fits the classical definition of a hormone being made in one organ, the mammalian kidney, being secreted into the bloodstream, and acting at another site, the blood-forming system, i.e., the bone marrow in adults. As a growth factor, it is required for red blood cell formation from more primitive cells of the hematopoietic system. In adult human beings, it regulates normal erythropoiesis, stimulating the production of approximately 2.5 million red cells per second.

I. HISTORY OF ERYTHROPOIETIN

The existence of erythropoietin (epo) was first suggested by Carnot and Deflandre in 1906. They described it (in current terminology) as a feedback regulator: when the number of red cells was below the normal setpoint, the mechanism to accelerate red cell formation was activated by secretion of epo; when the setpoint was regained, the mechanism was thought to turn off, keeping the number of red cells relatively constant. It is now known that normal red cell formation is regulated by continuous secretion of epo so that the formation of new red cells matches the loss of old cells. The system, however, can respond to a need for red cells, for example, due to blood loss or hypoxia, by increased secretion of epo and the

consequent increased formation of red cells. The experimental evidence published by Carnot and Deflandre was neither convincing nor reproducible and for almost 50 years the question of whether they had demonstrated that there was such a substance was in serious doubt. Its existence is now unquestionable.

The contemporary era of epo research began with the purification of human urinary epo and, 8 years later, the cloning of its gene. These advancements set the stage for a great increase in research and publications on epo: in the period from 1906 to 1976, there was an average of 16 papers on the subject published per year; from 1976 to 2000, this number was 237 per year. Of course, the greater part of this increase was due to the advent of recombinant DNA technology, making it possible not only to clone the gene and study its expression, but to produce epo in cells engineered to carry the transgene, harvest epo, and purify it in industrial amounts for clinical use. Much of the increase in the number of publications was associated with molecular biology studies and clinical application.

II. THE ERYTHROPOIETIN GENE

The human epo gene is on chromosome 7 (7pter-q22) and is a 2144 bp stretch of DNA. The structural gene consists of four introns, comprising 1562 bp, and five exons, totaling 582 bp. There is an 81 bp sequence coding for a signal peptide immediately downstream of the ATG start site. For the sequence of the signal peptide, see Fig. 1. Upstream of the signal sequence there do not appear to be any canonical promoter sequences. Downstream of the termination site, there is an important sequence defining the enhancer, hypoxia-response element, coding for hypoxia-inducible factor-1. There is little if any homology between the epo gene and the sequences of other mammalian genes except for these regulatory elements in the 3'-flanking region.

III. THE ERYTHROPOIETIN PROTEIN

The amino acid sequence of human epo (Table 1) shows it to consist of approximately 34% hydrophobic amino acids. There is little significant homology between the amino acid sequence of human epo and that of any other human hormone, cytokine, or growth factor.

H₂NMGVHECPAWLWLLSLLSLPLGLPVLGCOOH

FIGURE 1 Signal peptide sequence of human erythropoietin.



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