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Expression of extracellular superoxide dismutase by human cell lines

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Extracellular superoxide dismutase (EC-SOD) is the major SOD isoenzyme in extracellular fluids, but occurs also in tissues. The sites and characteristics of the synthesis of the enzyme are unknown. The occurrence of EC-SOD in cultures of a large panel of human cell lines was assayed by means of an e.l.i.s.a. Unlike the situation for the intracellular isoenzymes CuZn-SOD and Mn-SOD, expression of EC-SOD occurs in only a few cell types. None of the ten investigated suspension-growing cell lines produced EC-SOD. Among normal diploid anchorage-dependent cell lines, expression was found in all 25 investigated fibroblast cell lines, in the two glia-cell lines, but not in six endothelial-cell lines, two epithelial-cell lines or in two amnion-derived lines. Among neoplastic anchorage-dependent cell lines expression was found in 13 out of 29. EC-SOD was secreted into the culture medium by cell lines expressing the enzyme. The rate of EC-SOD synthesis varied by nearly 100-fold among the fibroblast lines and remained essentially constant in the individual lines during long-term culture. In the nine investigated cases, the secreted EC-SOD was of the high-heparin-affinity C type. It is suggested that tissue EC-SOD is secreted by a few well-dispersed cell types, such as fibroblasts and glia cells, to diffuse subsequently around and reversibly bind to heparan sulphate proteoglycan ligands in the glycocalyx of the surface of most tissue cell types and in the interstitial matrix.

INTRODUCTION

There are three mammalian superoxide dismutase (SOD, EC 1.15.1.1) isoenzymes. The two intracellular isoenzymes, CuZn-SOD [1] and Mn-SOD [2], are both synthesized by virtually all cell types in the body. The secretory extracellular SOD (EC-SOD) is the major isoenzyme in extracellular fluids, such as plasma, lymph [3] and synovial fluid [4], but has also been demonstrated in all investigated tissues in all investigated mammalian species [5,6]. In tissues it is the least predominant SOD isoenzyme. Still, 90–99 % of the EC-SOD in the body of mammals is found in the extravascular space of the tissues [5,6]. EC-SOD is a tetrameric Cu- and Zn-containing glycoprotein with a subunit molecular mass of 30 kDa [7,8]. Plasma EC-SOD in man [9] and other mammals [10] is heterogeneous with regard to affinity for heparin–Sepharose and can be divided into at least three fractions; A, without affinity; B, with intermediate; and C, with relatively high heparin affinity. EC-SOD A and B seem mainly to exist in the extracellular fluid phase *in vivo* [9,10]. EC-SOD C apparently exists in equilibrium between the fluid phase and its principal physiological ligand, heparan sulphate proteoglycan, located in the glycocalyx of cell surfaces and in the interstitial connective-tissue matrix [9–13].

The site(s) of synthesis of EC-SOD in the body is unknown. The present paper reports an analysis of EC-SOD synthesis in a large panel of cultured human cell lines. The type of EC-SOD synthesized was also analysed. It is shown that expression of EC-SOD occurs in only a few cell types, that the EC-SOD synthesized is secreted into the medium and that, in all investigated cell lines, it was type C.

MATERIALS AND METHODS

Cell culture

The various cell lines were cultured in appropriate media, containing 10 % (v/v) fetal-calf serum. The cell cultures were in general harvested 3–5 days after splitting, at which time the anchorage-dependent cells were mostly confluent. For EC-SOD analysis in anchorage-dependent cell lines, the medium was removed and the cells then loosened by incubation in PBS/10 mM-EDTA, followed by scraping, if necessary. The cell suspension was then centrifuged. The culture medium, the PBS/EDTA solution and the cell pellet were kept at −70 °C until assay. Suspension-growing cells were centrifuged, and the supernatant culture medium and the cell pellet kept separately at −70 °C. For analysis, the cell pellets were homogenized by sonication under cooling with ice in 50 mM-sodium phosphate, pH 7.4, containing 0.3 M-KBr, 10 mM-diethylenetriaminepenta-acetic acid, 0.5 mM-phenylmethanesulphonyl fluoride and aprotinin (100 kallikrein-inhibitory units/ml) (the last three additions to inhibit proteinases) (1 ml to cells from a 10 cm-diameter Petri dish). The homogenates were centrifuged (20000 *g*, 10 min) and analyses made on the supernatants.

SOD analysis

SOD enzymic activity was determined with the direct

Abbreviations used: (EC-)SOD, (extracellular) superoxide dismutase; PBS, phosphate-buffered saline (0.01 M-potassium phosphate/0.15 M-NaCl, pH 7.4).

Table 1. Expression of EC-SOD by cultures of human cell lines

EC-SOD was determined by means of an e.l.i.s.a. in culture media and cell homogenates of human cell lines as described in the Materials and methods section. For comparison, the CuZn-SOD and Mn-SOD activities of some of the cell fractions were also analysed. A blank space means 'not analysed' and a '0' that no EC-SOD could be detected by the e.l.i.s.a. procedure, which had a detection limit of about 0.03 ng/ml. All cell homogenates were analysed for EC-SOD before and after treatment with immobilized anti-(EC-SOD) antibody and the values presented are the differences between the two results.

Cell line and derivation	EC-SOD activity (ng/ml of medium)	EC-SOD activity (ng in medium/mg of cell protein)	EC-SOD activity (ng in cells/mg of cell protein)	CuZn-SOD activity (units/mg of cell protein)	Mn-SOD activity (units/mg of cell protein)
Anchorage-dependent					
Normal diploid					
Fibroblasts					
HEL (Embryonal lung)	0.14				
P21 (Embryonal lung)	1.6	42	7.9	147	33
MRC-5 (Embryonal lung)	3.6	46	12	110	6.2
Detroit 532 (Foreskin)	2.8				
FSV (Foreskin)	2.7				
AG 1518 (Foreskin)	0.66	15	3.4	71	5
AG 1523 (Foreskin)	6	142	9.7	58	1.9
BUD-8 (Skin)	0.46	6.1	1.7	82	8.5
HED (Skin)	2.8	40	5.2		
N-22 (gingiva)	2.9				
Skin cystic fibrosis ($n = 7$)	4.6±4.3				
Skin of controls ($n = 8$)	1.9±2.1				
Glia cells					
1508 CG	0.37	16	7.8	76	3.3
U1169 CG	0.8	34	1.1		
Endothelium, umbilical cord ($n = 6$)	0		0		
Epithelial cells, bucca ($n = 2$)	0				
WISH (amnion epithelial)	0	0	0	154	1.2
Amnion cells, fibroblastoid	0	0	0	55	1.2
Neoplastic					
Fibrosarcomas					
2149	0.45	5.3		125	7.9
2173	3.7	203	2.1	52	18
HT 1080	0	0	0	104	1.6
Charlie (simian-virus-40-transformed fibroblasts)	0	0	0	97	6.8
Malignant fibrous histiocytomas					
2197	8.1	119	18	293	6.5
2306	1.4	35	1.6	77	3
Malignant gliomas					
U138 MG	0	0	0		
U118 MG	3.1	51	4.0	75	6.5
178 MG	0	0	0	96	32
1240 MG	0	0	0	136	1.6
563 MG	0.54				
1231 MG	0.34				
31 L	0	0	0	225	7.9
4 H	0	0	0	231	2.9
2349 (epithelioid sarcoma)	1.3	87	6.0	52	8.0
Colon carcinomas					
LS174T	1.3	11	0.70	176	2.6
HT29	0	0	0	135	3.4
Colo 320 HSR	0	0	0	169	1.7
SW 116	0	0	0	85	6.2

Table 1. (*cont.*)

Cell line and derivation	EC-SOD activity (ng/ml of medium)	EC-SOD activity (ng in medium/mg of cell protein)	EC-SOD activity (ng in cells/mg of cell protein)	CuZn-SOD activity (units/mg of cell protein)	Mn-SOD activity (units/mg of cell protein)
Anaplastic thyroid cancer					
SW1731	0	0	0	108	14
C643	0.26	2.0	0.2	45	3.8
Hep G2 (hepatoma)	0				
SK-UT-1 (uterus leiomyosarcoma)	0.65	2.1	0.1	178	2.2
T24 (papillary ca urinary bladder)	0	0	0	93	3.6
RPMI 7272 (melanoma)	0.96				
U1285 (lung carcinoma)	0				
Saos-2 (osteogenic sarcoma)	2.9				
P31 (malignant mesothelioma)	0	0	0	164	2.1
HeLa 229 (cervix adenocarcinoma)	0	0	0	116	3.7
Suspension-growing					
Neoplastic					
Myelomas					
RPMI8226	0	0	0	123	1.4
MN601	0	0	0	145	1.3
MN60	0	0	0	182	2.4
U266	0	0	0	198	2.0
HL60 (myeloid leukemia)	0	0	0	111	2.2
Burkitt's lymphomas					
Raji	0	0	0	155	2.5
P3	0	0	0	162	2.4
Daudi	0	0	0	154	1.7
U937 (histiocytic lymphoma)	0	0	0	154	2.4
158 BU (B-cells Epstein–Barr-virus transformed)	0	0	0	91	2.1

spectrophotometric method employing KO_2 (potassium superoxide) [14] as modified [15]. Cyanide (3 mM) was used to distinguish between the cyanide-sensitive isoenzymes CuZn-SOD and EC-SOD and the resistant Mn-SOD. A unit corresponds to 8.3 ng of human CuZn-SOD, 8.6 ng of human EC-SOD and 65 ng of bovine Mn-SOD.

EC-SOD protein was determined with an e.l.i.s.a. [11]. The limit of sensitivity was about 0.25 ng/ml. To increase the sensitivity of the assay, samples were freeze-dried and then dissolved again in one-tenth the original volume of distilled water. The procedure resulted in a 9.2-fold concentration, pushing the detection limit down to about 0.03 ng/ml. All media lacking or showing a low EC-SOD content (as tested directly) were also tested after concentration. The procedure showed a nearly quantitative recovery of EC-SOD e.l.i.s.a. reactivity when small amounts of human EC-SOD [8] were added to media. The performance and specificity of the e.l.i.s.a. were tested in several ways. There was no cross-reaction with the bovine EC-SOD in the fetal-bovine serum. Freezing and thawing culture media with added EC-SOD ten times had no negative effect, nor did addition of heparin affect the reactivity. EC-SOD added to cell-culture media, physiological saline and the medium used for cell homogenization (see above) showed equal reactivity. Treatment of cell-culture media, apparently containing EC-SOD, with immobilized anti-(human EC-SOD) antibody [16] abolished all the e.l.i.s.a. reactivity. The only non-specific reaction noted was an apparent content of around 0.5–1 ng of EC-SOD/mg of cell protein in the cell homogenates (cf. Table 1 above). This reactivity was not removed by treatment with immobilized anti-(EC-SOD) antibody. Homogenates of cell lines secreting EC-SOD to the culture medium always contained more EC-SOD than did non-secreting lines. If these homogenates were treated with immobilized anti-(EC-SOD) antibody, most of the EC-SOD reactivity was lost, leaving around 0.5–1 ng/mg of protein. In Table 1, the EC-SOD content of cell homogenates presented is the difference between the amount found before and after treatment with immobilized anti-(EC-SOD) antibody.

Separation of culture media on heparin–Sepharose

Chromatography was performed at room temperature in 1 ml Heparin–Sepharose (Pharmacia Laboratory Separation Division, Uppsala, Sweden) columns, essentially as described previously [9], using 15 mM-sodium cacodylate/50 mM-NaCl, pH 6.50, as equilibration buffer and eluent. Bound EC-SOD was eluted with a 0–1 M-NaCl gradient. Fractions collected were assayed for EC-SOD by means of an e.l.i.s.a. Before application, the solvents of the culture media were 'exchanged' with the cacodylate equilibration buffer and the volume was halved by using a Filtron Omegacell membrane concentration system.

Protein analysis

For protein analysis, Coomassie Brilliant Blue G-250 was employed [17], standardized with human serum albumin.

RESULTS

Expression of EC-SOD by human cell lines

Table 1 collects the results of analysis of EC-SOD in culture media and homogenates of a large number of different human cell lines. For some of the cell lines we also analysed, for comparison, CuZn-SOD and Mn-SOD in the cell homogenates. Expression of EC-SOD is apparently not a common property of human cell lines. Among the few normal diploid cell types analysed, expression was found in fibroblasts and glia cells. No expression was found in endothelial cells or in epithelial cells. Among the neoplastic anchorage-dependent cell lines, expression was commonly found in cells derived from fibroblasts and in glioma-cell lines. Scattered findings of expression include two malignant fibrous histiocytomas, an epithelioid sarcoma, a melanoma, a uterus leiomyosarcoma, an osteogenic sarcoma, one of the four colon carcinomas and a thyroid carcinoma.

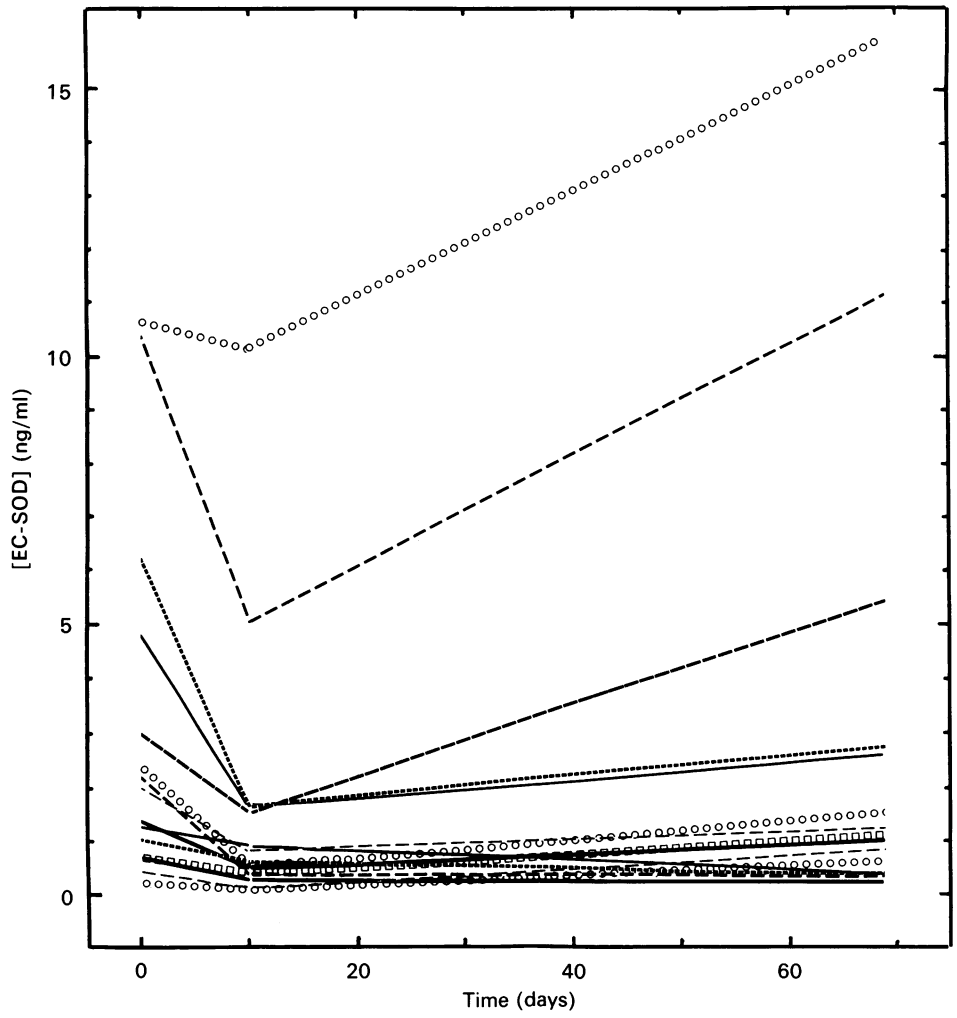

Fig. 1. EC-SOD expression by 15 skin fibroblast cell lines kept in continuous culture in parallel

The media were analysed for EC-SOD content on three occasions. The passage numbers of the cells were 11 ± 2 (S.D.) on the first occasion, the second and third samplings being done at 10 and 69 days after the first. The lines were split on average once a week and media were exchanged twice a week. The indicated media were obtained 4, 4 and 5 days respectively after the last medium exchange.

None of the ten investigated suspension-growing cell lines expressed EC-SOD.

After the initial finding of expression in fibroblasts, we subsequently surveyed a total of 25 different fibroblast lines derived from many different sources. We found expression of EC-SOD in all, although the amount expressed varied considerably [range 0.14–14 (mean 4) ng/ml in 3–5-day culture media]. The media of 15 skin fibroblast lines kept in continuous culture in parallel were analysed three times over a period of 60 days (Fig. 1). There were some general differences in EC-SOD content between the three occasions, because no attempts were made to keep the intervals between culture splitting, last medium exchange and sampling equal. Although the level of EC-SOD expression varied a lot between the cell lines, the relative expression of the individual cell lines remained remarkably constant.

Localization in culture of synthesized EC-SOD

As can be deduced from Table 1, most of the EC-SOD in the cultures was localized in the culture medium, but a sizeable portion also occurred in the cell fraction. EC-SOD C forms an equilibrium between the culture medium and heparan sulphate proteoglycans in the cell layer of cultured cell lines [12]. Addition of heparin to the medium abolishes the binding to the cell layer. Results of previous studies of such equilibria [12] suggest that the amount bound to the cell layer in the present investigations should be about 5% of the amount in the medium. Since the binding is rapidly reversible [12], much of the bound EC-SOD C should be lost to the EDTA-containing PBS used for removal of the cells from the dishes. Contents of the expected small magnitude were also found in these solutions. To distinguish further between remaining binding to extracellular heparan sulphate proteoglycan and localization to other sites in the cell fraction, cells were removed from replicate dishes of several cell lines with either PBS/EDTA or PBS/EDTA/heparin (150 i.u./ml). In none of the cases was there any significant difference in the EC-SOD content between non-treated and heparin-treated cell homogenates. The EC-SOD of the cell fraction is consequently not associated with extracellular heparan sulphate proteoglycan.

Turnover of EC-SOD in the medium of cell cultures

To determine the rate of turnover of EC-SOD C in cell cultures, recombinant human EC-SOD C [8] was added to the medium of cultured cells not expressing human EC-SOD. Samples were taken regularly and assayed for EC-SOD by e.l.i.s.a. At 3 and 30 μg/ml there was less than 10–15% loss of enzyme over a 4-day period in confluent cultures of the Chinese-hamster fibroblast line V-79 and Chinese-hamster ovary cells (CHO-Kl). At low concentrations (5 and 15 ng/ml) the enzyme disappeared with half-times of about 80, 100 and 140 h in V-79, CHO-Kl and MDBK (bovine kidney cells) cultures respectively. In control dishes with only medium and recombinant EC-SOD C incubated in parallel, there was no significant loss of EC-SOD C protein at any concentration. The nearly linear time course of EC-SOD appearance in the medium of EC-SOD-producing cell lines (Fig. 2) also indicates a slow enzyme turnover in the cultures.

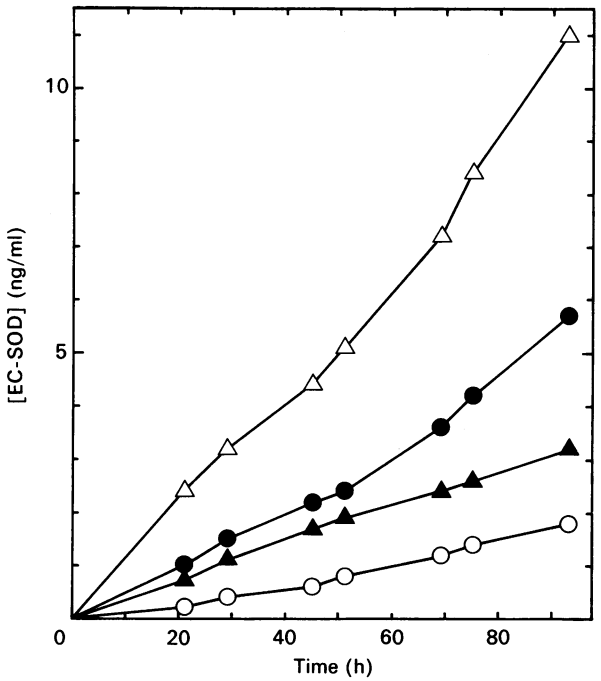

Fig. 2. Time course of EC-SOD appearance in cell-culture media

The cell lines were grown to confluence in dishes with 25 cm^2 bottom area. The media were removed, and 10 ml fresh medium was added. Aliquots, taken at indicated times, were analysed for EC-SOD content. CF 83 (△) and CF 93 (●) are skin fibroblast lines obtained from patients with cystic fibrosis, and K3 (○) is a control fibroblast line. U-1169 CG (▲) is a human glia-cell line.

Separation on heparin–Sepharose

To determine which subtype of EC-SOD was synthesized, culture media were separated on heparin–Sepharose. In all cases studied, LS 174T, U-118 MG, U-1169 CG, 2173 and five fibroblast lines, the EC-SOD was eluted from the heparin–Sepharose column at about 0.55 M-NaCl. The form of EC-SOD expressed by the cells was consequently the high-heparin-affinity type C.

DISCUSSION

EC-SOD is evolutionarily related to the CuZn-SODs, since the middle portion of the EC-SOD sequence shows strong homology with that part of the CuZn SOD sequence which defines the active site [18]. The human EC-SOD cDNA encodes an 18-amino-acid-long presequence which apparently is a signal peptide. CHO-Kl cells transfected with a vector containing the EC-SOD cDNA secreted recombinant human EC-SOD, lacking the presequence, to the medium [8]. The present investigation now shows that human cell lines that natively express EC-SOD also secrete the enzyme to the medium. Still a relatively large proportion (average around 11%) of the enzyme in 3–5-day cultures remained in the cell fraction. No significant part of that could be explained by binding to extracellular heparan sulphate proteoglycans. The turnover of EC-SOD C in the medium appears to be slow. Although the experiments were not designed to probe rigorously the characteristics

of the secretion, the findings thus suggest a long intracellular residence time of EC-SOD after synthesis. Other possibilities are binding to some specific intracellular site or to some extracellular slowly reversible non-heparin-sensitive ligand. A previous study of the binding of exogenous EC-SOD C to a large panel of cell lines [12] did not, however, suggest the existence of such a ligand on the exterior of the cells.

Whereas the intracellular isoenzymes CuZn-SOD and Mn-SOD are synthesized by virtually all mammalian cell types, expression of EC-SOD is not a common property. Among the normal diploid cell lines investigated, we found expression in fibroblasts and glia cells. Except for cell types derived from these two, there were only scattered findings of EC-SOD expression among the neoplastic anchorage-dependent cell lines. Whether these latter findings mean that the cells of origin in the body synthesize EC-SOD is, of course, uncertain. None of the investigated suspension-growing cell lines expressed EC-SOD. It is noteworthy that this absence of expression coincides with a minor to negligible surface binding of EC-SOD C by suspension-growing cell lines [12]. We found no expression of EC-SOD by human umbilical-cord endothelial cells, suggesting that the EC-SOD in the vascular tree is derived from tissue interstitial fluid, entering the vessels via lymph and diffusion through vessel walls. Synthesis by endothelial cells *in vivo* can, however, not be fully excluded, since in culture the cells are known to change the phenotype gradually and since endothelium in different areas of the vasculature differ in properties.

The content of Mn-SOD and especially CuZn-SOD varies only moderately between different cultured cell lines (Table 1; [19]). The EC-SOD content of synthesizing cultures, on the other hand, varies a lot, and even among skin fibroblasts there was a 100-fold variability. Since the rate of EC-SOD degradation in the medium is low, the difference in EC-SOD content should reflect differences in synthesis rates and not in rates of turnover. The rate of synthesis did not vary much over the time, and appeared to be a characteristic of each cell line (Fig. 1).

EC-SOD can be demonstrated in all tissues and accounts in man for about 1–2% of the total tissue SOD activity [5]. The enzyme is apparently secreted by only a few cell types. The present findings point to the possibility that fibroblasts are important sources of EC-SOD in peripheral tissues and glia cells in the central nervous system. However, the amount of EC-SOD expressed is small. An average 3–5-day fibroblast culture contains about 50 ng of EC-SOD (= 6 units of SOD activity)/mg of cell protein (Table 1). Human tissues contain on average 10 units (= 86 ng) of EC-SOD/mg of protein [5]. Since fibroblasts account for only a minor portion of tissue cells, it would seem that the observed EC-SOD synthesis rate is too low to account for the tissue EC-SOD content, unless the turnover of the enzyme is extremely slow. A putative cell type producing EC-SOD at a rate that would result in an EC-SOD content in a 3-day culture medium just below the detection limit of the e.l.i.s.a. procedure (0.03 ng/ml) would produce roughly 1 ng/3 days per mg of cell protein. Production at such rates by a major portion of the cells composing tissues can also not explain the tissue EC-SOD content. Maybe factors and conditions in tissues stimulate to higher EC-SOD synthesis than that which we find in standard cell culture. It is also possible that there are cell types in tissues, not encompassed in the present survey, that express more enzyme and account for a large part of tissue EC-SOD synthesis. Finally, the fibroblasts that proliferate from tissue biopsies may not be fully representative for the fibroblasts in the tissues and may represent a selection of low-expressing cells (cf. Fig. 1). The highest-expressing cell line in that Figure produces about 1000 ng/3 days per mg of cell protein, a productivity more compatible with the tissue EC-SOD content. EC-SOD C added to cultured anchorage-dependent cell lines forms an equilibrium between the medium and the cell layer [12]. The principal binding substance is heparan sulphate proteoglycan in the glycocalyx of the cell surfaces and in the interstitial matrix [12,13]. It is probable that tissue EC-SOD likewise binds to the surfaces of most or all cell types in the tissues and to the interstitial connective-tissue matrix. Since the binding is reversible [12], secretion by a few well-dispersed cell types should be sufficient for uniform tissue distribution of EC-SOD.

All the nine investigated cell lines secreted the high-heparin-affinity subtype EC-SOD C to the medium. Such secretion by tissue-derived cell types is in accord with the finding that EC-SOD in human tissues is mainly composed of forms with high heparin affinity (S. L. Marklund, unpublished work). The molecular background to the different EC-SOD subtypes is unknown. The amino acid compositions, antigenic properties [16] and molecular sizes on SDS/PAGE gels [7] are not with certainty different. In the *C*-terminal end of EC-SOD C there is a cluster of positively charged amino acid residues that probably confers the heparin-affinity [18,20]. Probably there are minor differences in this region due to differences at the gene level, differences in mRNA splicing, proteolytic processing within some secreting cell types or proteolytic activity in the extracellular space. The present finding of uniform EC-SOD C synthesis may be taken to support the last possibility.

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