Regulation by Cytokines of Extracellular Superoxide Dismutase and Other Superoxide Dismutase Isoenzymes in Fibroblasts*

(Received for publication, October 25, 1991)

Stefan L. Marklund‡
From the Department of Clinical Chemistry, Umeå University Hospital, S-901 85 Umeå, Sweden

The influence of cytokines on extracellular superoxide dismutase (EC-SOD) expression by human dermal fibroblasts was investigated. The expression was markedly stimulated by interferon-γ (IFN-γ), which varying between fibroblast lines stimulated or depressed by interleukin-1α (IL-1α), was immediately depressed by tumor necrosis factor-α (TNF-α), and markedly depressed by transforming growth factor-β (TGF-β). TNF-α, however, enhanced the stimulation by a high dose of IFN-γ, whereas TGF-β markedly depressed the stimulations given by IFN-γ and IL-1α. The ratio between the maximal stimulation and depression observed was around 30-fold. The responses were generally slow and developed over periods of several days. There were no effects of IFN-α, IL-2, IL-3, IL-4, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor, human growth hormone, Escherichia coli lipopolysaccharide, leukotriene B₄, prostaglandin E₂, formylmethionyleucylphenylalanine, platelet-activating factor, and indomethacin. The cytokines influencing the EC-SOD expression are also known to influence superoxide production by leukocytes and other cell types, and the EC-SOD response pattern is roughly compatible with the notion that its function is to protect cells against extracellular superoxide radicals. The results show that EC-SOD is a participant in the complex inflammatory response orchestrated by cytokines.

The CuZn-SOD activity of the fibroblasts was not markedly depressed by any of the cytokines, whereas the Mn-SOD activity was depressed by TGF-β. TNF-α, IL-1α, and IFN-γ stimulated the Mn-SOD activity, as previously known, and these responses were reduced by TGF-β. The different responses of the three SOD iso-enzymes illustrate their different physiological roles.

Extracellular superoxide dismutase (EC-SOD),¹ EC 1.15.1.1) is a secretory tetrameric copper- and zinc-containing glycoprotein (1, 2). It is the major SOD isoenzyme in extracellular fluids such as plasma, lymph (3–5), and synovial fluid (6), but occurs also in tissues (7, 8). Of the total amount of EC-SOD in the body of mammals, 90–99% can be calculated to exist in the interstitial space of tissues (3, 4, 5, 7, 8). EC-SOD is thus primarily a tissue enzyme. Apart from being secretory, the most distinguishing property of EC-SOD is its affinity for heparin/heparan sulfate (1, 9, 10). In vivo, EC-SOD is apparently to a major part sequestered by heparan sulfate proteoglycan in the glyocalyx cell surfaces and in the connective tissue matrix (4, 5, 10, 11). Although the overall tissue concentration of EC-SOD in most tissues is lower than those of the intracellular isoenzymes CuZn-SOD and Mn-SOD (7, 8), the small volume of the interstitial space means that the local concentration of EC-SOD will be considerable. EC-SOD thus forms the major physiological defence against superoxide radicals released to the extracellular space. Unlike the situation with the intracellular isoenzymes CuZn-SOD and Mn-SOD, expression of EC-SOD occurs in only a few cell types (12). A comprehensive study of human cell lines indicated that fibroblasts may be an important source of EC-SOD in the body (12).

The regulation of EC-SOD synthesis is unknown. Phagocytes activated during the inflammatory response are likely major sources of substrate for EC-SOD. Furthermore, fibroblasts are known to respond to a variety of cytokines involved in inflammation, growth, and wound healing. We therefore decided to study the effects of various cytokines involved in the regulation of inflammation and immune reactions on fibroblast EC-SOD synthesis. We here present evidence that such cytokines fundamentally influence EC-SOD expression.

**EXPERIMENTAL PROCEDURES**

Cell Culture and Regulation Experiments—Human skin fibroblast lines were initiated from skin punch biopsy specimens obtained from healthy volunteers (ages 16, 3, 19, 9, and 35 years for the lines K1, K3, K4, K25, and C3, respectively), using Ham's F10 with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, and 2 mM glutamine as medium. The lines were used between the 10th and the 20th passages.

For synthesis regulation experiments the cells were mostly seeded into 12-well culture plates, 3.80-cm² bottom area, and grown into near confluence. To suppress growth and reduce interference from serum factors, the medium was exchanged twice to medium with 0.5% fetal calf serum about 20 h before the start of the experiments. The experiments were started by exchange to 0.5-mL medium with 0.5% fetal calf serum containing indicated concentrations of cytokines or only medium with 0.5% fetal calf serum (controls). Every 24 h the media were collected and replaced with fresh media containing cytokines. At the end of the experiments, mostly after 4 days, the media were collected and the wells were washed three times with 0.15 M NaCl. To collect and homogenize the cells, 0.5 ml of ice-cold 50 mM sodium phosphate, pH 7.4, containing 0.3 M KBr, 10 mM diethylenetriamine pentaacetic acid, 0.5 mM phenylmethylsulfonyl fluoride, and 100 KIU/ml aprotinin (the latter three additions to inhibit proteases) was added to the wells. After sonication in the wells, the plate bathing in ice water, the homogenates were centrifuged (20,000 × g × 10 min) and the supernatants were collected for analysis. All

* The study was supported by Grant 9204 from the Swedish Natural Science Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom reprint requests should be addressed. Tel.: 90-101239; Fax: 90-117296.

‡ Umeå, Sweden

1 The abbreviations used are: EC-SOD, extracellular superoxide dismutase; CuZn-SOD, copper/zinc superoxide dismutase; Mn-SOD, manganese superoxide dismutase; IFN-γ, interferon-γ; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; GM-CSF, granulocyte-macrophage colony-stimulating factor; FMLP, formylmethionyleucylphenylalanine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
samples were kept at −80 °C before assay.

Incorporation of[^35]S]Methionine into Protein—Fibroblasts cultured in 12-well culture plates (3.8 cm²) were washed three times with methionine-free culture medium. Thereafter, 0.5 ml of methionine-free medium containing 0.5% fetal calf serum and L-[^35]S]methionine (0.18 MBq/ml) was added and the cells were incubated at 37 °C for 1 h. The medium was then put on ice and the cells were washed three times with 0.15 M NaCl. The cells were then sonicated in 0.5 ml of buffer, as described above for the regulation experiments. To 100 μl of homogenate + 25 μl of fetal calf serum was added 2 ml of ice-cold 10% trichloroacetic acid containing 10 mM methionine. The precipitate was collected by centrifugation (20,000 × g, 0.5 min) and was washed three times with the methionine-containing trichloroacetic acid. The precipitate was thereafter dissolved in 10 ml of scintillation fluid and counted in a β-counter. Addition of 0.18 mM cycloheximide to the medium reduced the incorporation by 97%.

SOD Analysis—EC-SOD protein was determined in cell culture media and cell homogenates with an enzyme-linked immunosorbent assay (ELISA) (11). The limit of sensitivity was about 0.25 ng/ml. There was no cross-reaction with the bovine EC-SOD in the fetal calf serum. SOD enzymic activity in cell homogenates was determined with the direct spectrophotometric method employing KO2 (13) as reducing agent. The absorbance was measured at 440 nm using a coupled assay for CuZn-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. The CuZn-SOD activity was obtained as total activity minus activity in the presence of 5 mM cyanide, minus units of EC-SOD as calculated from ELISA and KO2 assay. The KO2 assay is performed at high pH and relatively high superoxide concentration. The common xanthine oxidase-CyC assay (15) is performed under more physiological conditions, neutral pH, and low superoxide concentration. One unit in the KO2 assay corresponds to 0.24 units of human CuZn-SOD and EC-SOD in the “xanthine oxidase” assay and to 0.24 units of Mn-SOD. The KO2 assay is thus 10 times more sensitive for CuZn-SOD and EC-SOD activity than for Mn-SOD activity.

Protein and DNA Analysis—For protein analysis, Coomasie Brilliant Blue G-250 was employed (16), standardized with human serum albumin. DNA was determined with fluorometry as a complex with (2-12-(4-hydroxyphenyl)-6-benzimidazolyl-6-1-(methyl-4-piperazyl)-benzimidazol 3HCl (Hoechst 33258) (17) using calf thymus DNA as standard.

Materials—The following recombinant human cytokines were obtained from Genzyme Corp., Boston, MA; IFN-γ (2.5 × 10⁶ units/mg), TNF-α (2 × 10⁶ units/mg), IL-1 (10⁶ units/mg), IL-3 (10⁶ units/mg), IL-4 (10⁶ units/mg), IL-6 (10⁶ units/mg), IL-8, GM-CSF (monocyte-derived). Ultrapure TGF-β from human platelets was purchased from Calbiochem Corp. Recombinant human IFN-α-2b (2 × 10⁶ units/mg) was obtained from Schering Corp, Kenilworth, NJ, recombinant human IL-2 (2 × 10⁶ units/mg) from Boehringer Mannheim GmbH, Mannheim, Germany, recombinant human growth hormone 101 from KABI-Pharmacia AB, Stockholm, Sweden. Iodemacin, Eucharchia cell, 055 B5 lipopolysaccharide, platelet-activating factor, leukotriene B4, and FMLP were obtained fromSigma. F-10 cell culture medium and fetal calf serum was purchased from Flow Ltd., Irvine, Scotland, and GIBCO Ltd., Scotland, respectively.

RESULTS

Effects of Interferon-γ, Interleukin-1α, Tumor Necrosis Factor-α, and Transforming Growth Factor-β—Fig. 1A reveals the effect of varying doses of interferon-γ (IFN-γ) on EC-SOD expression of a fibroblastic cell line. A stimulation of expression is seen which is half-maximal at 5 units/ml and near-maximal at 50 units/ml. The response to the IFN-γ is slow, and appears to be maximal relative to controls after 2–3 days. There is also a similar increase in EC-SOD content of the cells homogenized at the end of the experiment. The stimulation of EC-SOD synthesis is not due to stimulation of cell growth, since there was no difference between controls and IFN-γ-treated cultures with regard to protein and DNA content in the cell layer.

Fig. 1B shows the response of a fibroblast line to interleukin-1α (IL-1α). As with IFN-γ the response is slow. Already at 0.05 unit/ml there was some stimulation of EC-SOD expression, it was maximal at 0.05 unit/ml and essentially lost at 5 and 50 units/ml. The response, however, differed much between fibroblast lines and in some cases only depression of synthesis was seen (see Fig. 3 below). The effect on the EC-SOD content of the cell homogenates was similar to that seen in the culture media. The IL-1α treatment did not influence cell growth, since the DNA and protein contents of the cell homogenates were not affected.

Tumor necrosis factor-α (TNF-α) mainly depressed the expression of EC-SOD, Fig. 1C. The effect was not apparent until the 3rd day, and was significant at 1 ng/ml and maximal at about 30 ng/ml. The full effect of TNF-α was apparently not expressed after 4 days. As with the other cytokines there was no effect of TNF-α on the protein and DNA contents of the cell homogenates.

TGF-β induced a late marked progressive depression of EC-SOD expression already seen at 0.005 ng/ml and maximal at about 25 ng/ml, Fig. 1D. The depression of EC-SOD synthesis was also apparent in the cell homogenates. The TGF-β treatment resulted in a slight reduction in the protein and DNA content of the cell homogenates, which, however, was much less extensive than the depression of EC-SOD synthesis.

The effect of the cytokines on EC-SOD expression was late and appeared in some cases to be progressive in the experiments shown in Figs. 1A to 1D. We therefore followed the effect of the cytokines on two fibroblast lines, K1 and K3, for a period of 11 days (Fig. 2). The response of the two lines was, in principle similar, the difference being that the line not shown in the figure, K1, responded to both IL-1α concentrations with depression of EC-SOD expression (cf. also Fig. 3). The stimulation by IFN-γ peaked after 5 days and thereafter declined and approached that of the controls at the end of the experiment. IL-1α at 0.5 unit/ml stimulated expression for the first 4 days but became thereafter inhibitory. 50 units/ml resulted in a late inhibition plateauing after 7–8 days at about 20% of the controls. TNF-α inhibited the EC-SOD expression with plateaus reached at 10–15% of the controls at about day 8. TGF-β was distinctly most depressing and reduced the EC-SOD synthesis of the cell lines down to 3–4% of the controls at day 8. The EC-SOD contents of the cell homogenates at the end of the experiment were reduced by 1.5 cytokines roughly to the same extent as were the contents of the culture media. The DNA and protein contents of the cell homogenates at the end of the experiment were, however, only minimally affected by the cytokines, showing that the effects observed were not due to influences on cell growth.

Skin fibroblast lines are individuals and differ in responses to cytokines and other factors. To ascertain the generality of our findings we therefore studied the responses of five fibroblast lines to various doses of cytokines. Data for maximally effective doses are presented in Fig. 3. To compare the response of EC-SOD with those of the other SOD isoenzymes, we also determined CuZn-SOD and Mn-SOD in all cell homogenates.

IFN-γ stimulated EC-SOD expression in all lines, in most cases 2- to 4-fold. The response to IL-1α was variable. At 0.5 unit/ml two lines were stimulated, one unaffected and two depressed. The higher concentration, 50 units/ml, resulted in more depression, but one line remained stimulated. These patterns are reproduced when the experiments are repeated. TNF-α inhibited the EC-SOD expression in all fibroblast lines. TGF-β depressed EC-SOD expression by all lines and was by far the most effective in that respect.

The CuZn-SOD activity of the fibroblasts was not significantly affected by any of the cytokines. The Mn-SOD activity
**Cytokines Regulate Extracellular SOD Synthesis**

**Fig. 1.** Effects of IFN-γ (A), IL-1α (B), TNF-α (C), and TGF-β (D) on fibroblast EC-SOD synthesis. Fibroblasts (line K3) were cultured in 3.8-cm² wells and culture media (0.5 ml) containing the cytokines at indicated concentrations were exchanged daily for 4 days. At the end of the experiment the cells were homogenized and analyses were made on culture media and cell homogenates as described under “Experimental Procedures.” The data presented are the means of results from two wells.

**Fig. 2.** Long-term effects of cytokines on EC-SOD expression. A fibroblast line (K3) was cultured in 3.8-cm² wells and culture media (0.5 ml) containing the cytokines at maximally effective doses were exchanged daily for 11 days. At the end of the experiment the cells were homogenized and analyses were made on culture media and cell homogenates as described under “Experimental Procedures.” The data presented are the means of results from two wells.

was increased by IFN-γ about 2-fold. The effect was half-maximal at 50 units/ml and maximal at 500 units/ml. IL-1α markedly increased the Mn-SOD activity in all cell lines. The effect was half-maximal already at 0.005 unit/ml and near maximal at 0.05 unit/ml (data not shown). TNF-α increased the Mn-SOD activity even more than IL-1α. The effect was half-maximal at 0.1 ng/ml and maximal at 1 ng/ml (data not shown). The major part of the Mn-SOD induction by IL-1α and TNF-α is, however, lost upon prolonged exposure to the cytokines, and the effect of IFN-γ became rather inhibitory (see Fig. 2). Finally, in four of the five fibroblast lines, TGF-β depressed the Mn-SOD activity.

**Time Course of Cytokine Effects on Fibroblasts**—Figs. 1, A–D, and 2 show that the effects of cytokines on fibroblast EC-SOD secretion develop over a period of several days. However, the parameters analyzed in the cells were only studied at the end of the 4-day experiments. We therefore also harvested cells at the start of the experiment, and then each day after exposure to IFN-γ, TNF-α, IL-1α, and TGF-β at maximally effective doses. Some of the results are collected in Fig. 4. Exposure to IFN-γ led to a continuous rise in the cell content of EC-SOD and the amount secreted rose in parallel. There was also a lower continuous rise in the amount of EC-SOD in cells and medium in control cultures. After exposure to TGF-β the cell and media contents of EC-SOD declined roughly in parallel. The marked rise in Mn-SOD induced by TNF-α and IL-1α also developed continuously over the tested period. The cellular content of CuZn-SOD did not change, whereas there was a minor rise, about 30%, in Mn-SOD content over the test period in control cultures (data not shown). The serum starvation in the experimental model resulted in some parallel
FIG. 3. Effects of cytokines on EC-SOD (EC), CuZn-SOD (CuZn), and Mn-SOD (Mn) synthesis by five different fibroblast lines. The fibroblast lines (Δ, K1; ●, K3; ○, K4; △, K25; and □, C9) were cultured in 3.8-cm² wells and culture media (0.5 ml) containing the cytokines at indicated maximally active concentrations (IL-1α also at an intermediate concentration) were exchanged daily for 4 days as described under “Experimental Procedures.” EC-SOD was determined in the culture media of the last day and CuZn-SOD and Mn-SOD were determined in the cell homogenates at the end of the experiment. The figure shows the activities as percentage of controls and before that calculation all data were divided with the protein contents of the cell homogenates to compensate for any differences in cell mass. The data shown are the means of the results for two wells. The amounts of enzyme found in the controls were for CuZn-SOD 148 ± 56 (S.D.) units per mg of cell protein, for Mn-SOD 4.7 ± 2.0 (S.D.) units per mg of cell protein, and for EC-SOD 50 ± 21 (S.D.) ng of EC-SOD in the last 24-h culture medium per mg of cell protein.

FIG. 4. Time course in changes in cells and media induced by cytokines. Fibroblasts (line K3) were cultured in 3.8-cm² wells and culture media (0.5 ml) with added cytokines (IFN-γ, 500 units/ml; TNF-α, 30 ng/ml; IL-1α, 50 units/ml; TGF-β, 25 ng/ml) were exchanged daily for 4 days. At the start of the experiment and each following day, cells (two wells for each experimental group) were collected and homogenized. Analyses were made on both culture media and cell homogenates. The results are presented as percentage of initial contents in cell homogenates and content in the first day media of control cultures, and before that calculation the data were divided with the protein contents of the cell homogenates (except the results for protein and DNA). ○, ●, EC-SOD in cells and media, respectively, in control cultures; Δ, △, EC-SOD in cells and media, respectively, exposed to IFN-γ; ■, ■, EC-SOD in cells and media, respectively, exposed to TGF-β; □, □, Mn-SOD in cells exposed to TNF-α and IL-1α, respectively; ○, ●, cell protein and DNA in control cultures. The EC-SOD content of cells exposed to TGF-β were at days 3 and 4 too low to analyze by ELISA.

loss of protein and DNA, which mainly occurred during the first day. The results with a second tested fibroblast line, K25, were essentially identical.

Effects of Combinations of Cytokines—TNF-α dose-dependently enhanced the stimulatory effect on EC-SOD synthesis of a maximally effective dose of IFN-γ (Fig. 5). After the second day, TNF-α depressed the effect of IFN-γ. When TNF-α was added to a low dose of IFN-γ, 5 units/ml, only a dose-dependent depression of the stimulation of EC-SOD synthesis was seen (data not shown).

The effect of other combinations were less remarkable, why the data are not shown. TGF-β efficiently depressed the stimulation by 500 units/ml IFN-γ: the effect began at about 0.05 ng/ml, was half-maximal at 0.5 ng/ml, and at 5 ng/ml the EC-SOD secretion was the same as that found with TGF-β alone. A similar pattern was observed between TGF-β and stimulatory concentrations of IL-1α. Between various concentrations of IL-1α and IFN-γ only additive effects (or “subtractive” when IL-1α was depressing) were observed. IL-1α at various concentrations could not enhance the stimulation by a maximally active dose of IFN-γ, 500 units/ml. The depressing effects of intermediately active doses of TNF-α and TGF-β were additive, but TNF-α did not enhance the depression seen with maximally active doses of TGF-β, ≥5 ng/ml.

None of the combinations influenced the CuZn-SOD activity of the cells. However, in addition to the direct effects on Mn-SOD described above, TGF-β also approximately halved the stimulation of Mn-SOD activity induced by TNF-α and IL-1α and the effect of IFN-γ was abolished.

Effect of Cytokines on Protein Synthesis—At the end of all experiments the protein and DNA contents of the cell layers...
were analyzed to exclude that the modulating effects of the cytokines on EC-SOD expression were secondary to major effects on cell growth or to toxicity. To further ascertain that the effects on EC-SOD expression were not secondary to general effects on protein synthesis, the incorporation of [35S]methionine into trichloroacetic acid-precipitable protein was studied at the end of 4-day experiments with two fibroblast lines. Relative to controls, the incorporations in the K1 and K3 cell lines amounted to 133 and 91% with 500 units/ml IFN-γ, 107 and 106% with 0.5 unit/ml IL-1α, 101 and 93% with 50 units/ml IL-1α, 136 and 142% with 30 ng/ml TNF-α, and 175 and 165% with 25 ng/ml TGF-β (means of results of three wells). The protein and DNA contents of the cell cultures were only minimally affected by the cytokines in the experiment.

**Other Cytokines Involved in Inflammation and the Immune Response**—The responses with regard to EC-SOD expression were tested in three fibroblast lines and were carried out as outlined in Figs. 1A to 1D. None of the cell lines responded to IFN-α (50 and 500 units/ml), IL-2 (1000 units/ml), IL-3 (3, 10, 30 ng/ml), IL-4 (1, 10, 40 ng/ml), IL-6 (50, 500, and 5000 units/ml), IL-8 (1, 4, 15, 60, 160 ng/ml), GM-CSF (0.3, 1, 3, 10, 30 ng/ml), and to human growth hormone (10, 30, 100, 300, 1000 ng/ml). None of these cytokines influenced the CuZn-SOD and Mn-SOD activities of the cells.

**Effects of Other Factors Involved in the Inflammatory Response**—Indomethacin (1 mM) added to culture media as described for cytokines under “Experimental Procedures” did not influence the EC-SOD expression by two tested fibroblast lines (K1, K3), nor did it influence the effects on expression by IFN-γ, IL-1α, TNF-α, and TGF-β. None of the cell lines responded to prostaglandin E2 (0.01, 0.1, and 1 μM), E. coli lipopolysaccharide (1, 10, 100, and 1000 ng/ml), leukotriene B4 (0.1, 0.5, 2, and 4 μM), platelet-activating factor (4, 20, 100, 500 nM), and FMLP (0.16, 0.5, and 1.5 μM). Nor did any of these factors influence the CuZn-SOD and Mn-SOD activities of the cells.

**DISCUSSION**

The present results show that the expression of EC-SOD is profoundly influenced by cytokines involved in the inflammatory response.

IFN-γ markedly enhanced expression of EC-SOD. The effect is not shared by other interferons since IFN-α was without effect. IFN-β which acts via the same receptor as IFN-α (18) should also not influence EC-SOD expression. The effect of IL-1α was variable. At low doses some lines were stimulated, whereas higher doses were more inhibitory. After prolonged exposure to a high IL-1α concentration, depressions of EC-SOD expression down to about 20% of the controls were noted. TNF-α exerted a late progressive depressive action on EC-SOD expression down to about 10−15% of controls (Figs. 1B and 2). TGF-β markedly progressively depressed EC-SOD expression down to about 3−4% of controls. The maximal ratio between stimulation (IFN-γ) and depression (TGF-β) observed was considerable, around 30-fold (Fig. 2).

As to interactions between the cytokines, TNF-α markedly dose-dependently enhanced the stimulation by a maximally active dose of IFN-γ (Fig. 5); whereas a dose-dependent depression of the effect of an immediately active dose of IFN-γ was seen. The enhancement may be due to an up-regulation of IFN-γ receptors by TNF (19). IL-1α which in several aspects influence fibroblasts similarly to TNF-α, did not exert such effects but rather interacted additively/subtractively with intermediate and high doses of IFN-γ. No stimulation of the effect of a high dose of IFN-γ was seen.

TGF-β efficiently abrogated stimulatory effects of IFN-γ and IL-1α.

The fibroblasts responded slowly to the cytokines with regard to EC-SOD secretion. It is apparent from Fig. 4 that this is due to a slow response in the synthesis rates of EC-SOD. The slow increases and decreases in cell content of EC-SOD is not due to accumulation or to loss of enzyme, since the amounts of EC-SOD found in cells correspond only to about half the amount secreted over a 24-h period (cf. Fig. 1), and the secretion rates increased and decreased in parallel with the cell contents (Fig. 4). The ratio between amounts of enzyme in cells and amounts secreted were essentially independent of the various treatments (Figs. 1, 2, and 4), indicating that the intracellular transit times were not affected by the cytokines. The control cells increased their secretion of EC-SOD continuously (Figs. 1, 2, and 4). We have at present no explanation for the phenomenon, which, however, may be related to the quiescent state induced by the serum starvation. Uptake and degradation of EC-SOD should occur only to minor extent in the present model since the media were exchanged every 24 h and the half-life of EC-SOD in the medium over very dense cell cultures mostly exceeds 100 h (12). The increases in Mn-SOD content induced by TNF-α and IL-1α also developed over several days (Fig. 4), but since the intracellular turnover rate of Mn-SOD is unknown, no assertion as to the development of the synthesis rate can be made.

The effects of the cytokines were not due to major influences on proliferation or general metabolism of the cells, since there were only minor differences versus control in cell protein and DNA at the end of the experiments. Furthermore, IFN-γ and IL-1α did not significantly influence the protein synthesis rate of the cells, whereas TNF-α and especially TGF-β stimulated the synthesis.

The question of whether the observed effects of cytokines were direct or indirect was addressed to some extent. The concentration ranges in which the effects of cytokines were seen are similar to the concentrations reported effective in many other models. The media with test substances were exchanged each day thereby avoiding problems with depletion or degradation of cytokines, and influences on EC-SOD expression by substances formed by cytokine-stimulated fibroblasts. IL-6, which is formed by fibroblasts stimulated by TNF-α and IL-1α (20, 21), had no effect on EC-SOD expression. Prostaglandin E2 and indomethacin in itself or added to the cytokines did not influence EC-SOD expression; thus the stimulation of prostaglandin synthesis by TNF-α and IL-1α (22) were not responsible for effects on EC-SOD expression. Since lipopolysaccharide was without effect, influences of endotoxin contamination can be ruled out.

Most of the cytokines tested have been reported to influence the superoxide radical production in tissues. Thus IFN-γ (23, 24) and IL-1 (25) enhance superoxide production by human monocytes/macrophages, whereas IFN-α and TNF-α lack effect (24). However, with mouse macrophages stimulation of superoxide production by TNF-α has been reported (26). Neutrophil leukocytes are primed for superoxide production by TNF-α (27), and IFN-γ augments the effect of TNF-α (28). The oxidant production by eosinophils is enhanced by IFN-γ (29) and TNF-α (29, 30). IL-6 primes neutrophils and monocytes to superoxide production (31), and IL-8 exerts both a strong chemotactic effect on neutrophils and activates their superoxide production (32). GM-CSF enhances superoxide production by monocytes (33) and neutrophils (34). Growth hormone primes macrophages for superoxide production (35), whereas IL-4 inhibits (36). A variety of cell types,
cytosolic CuZn-SOD, and the Mn-SOD located to the mitochondrial matrix, illustrate their different physiological roles.

Acknowledgments—The skillful technical assistance of K. Hjertkvist, A. Öberg, and E. M. Öhman is gratefully acknowledged as is the gift of the fibroblast lines from Dr. O. Ceder, Department of Pediatrics, Umeå University Hospital.

REFERENCES