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Transforming Growth Factor-β Does Not Alter Interleukin-1 Expression in Cultured Human Macrophages

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Transforming growth factor-β (TGFβ) is a growth modulator that stimulates the growth of fibroblastic cells but inhibits the growth of cells of epithelial origin. TGFβ also influences the production of extracellular matrix proteins, and of proteases and the type 1 plasminogen activator inhibitor (PAI-1) by cultured cells. TGFβ appears also to have various immunoregulatory effects, suppressing both T- and B-cell activities. It has been proposed that it might increase the expression of interleukin-1 (IL-1) mRNA in cultured human monocytes, thus potentiating immune functions. To analyze the role of TGFβ in IL-1 production we have now quantitated the effect of this factor on the production of biologically active IL-1 as well as IL-1β mRNA expression. The effect of TGFβ on IL-1 production optimally activated with bacterial lipopolysaccharide (LPS) was also studied. It was found that IL-1 activity and mRNA levels were rapidly elevated by LPS but not by TGFβ. Culture fluids from monocytes treated with TGFβ alone or with TGFβ plus LPS inhibited the proliferation of the test thymocytes. After gel filtration, the media from TGFβ-treated cultures showed no activity in the molecular weight area of IL-1 (approx. 15 kD), while the supernatants from TGFβ plus LPS-induced cells contained IL-1 activity in these fractions, the magnitude of which was, however, at the same level as in the culture fluids derived from cells stimulated with LPS alone. Thus our results show that the TGFβ used was biologically active but they provide no evidence for TGFβ in the regulation of IL-1 production in human monocytes.

Key words: human monocytes, cytokine interactions, immune suppression, monokine, endotoxin

Malignant transformation of cultured cells is often associated with the production of specific growth factors [1–3], some of which have been termed transforming growth factors (TGFs) because of their ability to induce anchorage-independent growth in nonmalignant cells. TGFβ is mainly responsible for the induction of anchorage-independent growth in fibroblastic cells, either alone [4], or in combination with epidermal growth factor (EGF) or TGFα [5]. TGFβ acts presumably as an indirect mitogen for fibroblastic cells grown in adherent cultures by inducing the expression of the c-*sis* proto-oncogene [6]. TGFβ also induces the production of platelet derived growth factor (PDGF)-A chains by several cell types including monoblastic leukemia

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cells [7]. TGFβ appears, however, to have a dual role in the regulation of cell growth: while stimulatory for cells of mesenchymal origin, it is a potent growth inhibitor for cultured epithelial cells [8]. The mechanism of this inhibitory activity of TGFβ is unclear at present.

TGFβ appears to have important functions in the regulation of the formation and degradation of the extracellular matrices of cultured cells. It elevates the production of fibronectin and procollagen, resulting in enhanced formation of the matrix [9], and modulates extracellular proteolytic activity by regulating both the secretion of plasminogen activators [10] and the endothelial-type plasminogen activator inhibitor PAI-1 [11].

Interestingly, TGFβ also performs various functions in the regulation of immunological responses, suppressing both T- and B-cell functions [12,13]. TGFβ also prevents the cytolytic activity of natural killer cells and diminishes their responses to α-interferon [14]. However, it has recently been proposed that TGFβ increases the level of IL-1 mRNA in human monocytes [15]. If this activation also results in production of significant levels of IL-1, it might counteract the immunosuppressive function of TGFβ, because IL-1 is a factor that is required for (or at least greatly facilitates) T-cell activation and possibly also enhances the maturation of B cells [16]. To analyze the role of TGFβ in IL-1 production, we have now quantitated its effect on IL-1β mRNA expression and on IL-1 production in cultured human monocytes. Additionally, the effect of TGFβ on IL-1 production that was optimally activated by bacterial lipopolysaccharide (LPS) was estimated.

MATERIALS AND METHODS

Reagents

TGFβ (form 1) was obtained from R&D Systems (Minneapolis, MN). Lipopolysaccharide (LPS; *E. coli* 026:B6) was purchased from Difco Laboratories (Detroit, MI).

Cell Cultures

Mononuclear cells were isolated from leukocyte-rich buffy coats by centrifugation in Ficoll-Isopaque (Pharmacia, Uppsala, Sweden). After washing, the cells were resuspended at a concentration of 10^7 cells/ml in RPMI-1640 (Flow Laboratories, Irvine, Scotland) medium containing 10% heat-inactivated human AB serum (The Finnish Red Cross Blood Transfusion Service, Helsinki, Finland), 10 mM Hepes buffer, 2 mM L-glutamine, and antibiotics (complete medium). The cells were incubated in plastic tissue culture flasks at 37°C for 1 h, and then the nonadherent cells were removed by vigorous pipetting with warm medium. Subsequently the adherent cells were harvested by incubating the cells in cold phosphate-buffered saline and using a rubber policeman. Of these, about 80–95% were monocytes.

The monocytes were seeded at a density of 1×10^6 cells in 1 ml of complete medium in 24-well tissue culture plates (Costar, Cambridge, MA), and the cultures were stimulated with the indicated combinations of TGFβ and LPS. After 24 h the culture supernatants were harvested and clarified by centrifugation. The samples were either fractionated by fast protein liquid chromatography (FPLC) gel filtration or kept at −20°C until tested for IL-1 activity.

For RNA isolations, each of the buffy coat preparations used was divided into

the experimental groups indicated in 90-mm-diameter Petri dishes, nonadherent cells were removed, and the adherent cells were then directly stimulated. The cells were collected at the times indicated, and RNA was isolated (see below).

In one experiment monocytes were purified using a discontinuous Percoll density gradient [17]. These were cultivated at a density of 1×10^6 cells/ml in 90-mm-diameter Petri dishes and stimulated as described in Results.

FPLC Gel Filtration

Samples of the culture medium (250 μl) were fractionated using FPLC with a prepacked Superose 12 column (Pharmacia). The column was eluted with PBS (flow rate 0.5 ml/min), and 0.5-ml fractions were collected. These fractions were then tested for IL-1 activity. The calibration markers for the column were ovalbumin (mol. wt. 45,000), α-lactalbumin (mol. wt. 14,200) and aprotinin (mol. wt. 6,500) (Sigma Chemical Co., St. Louis, MO).

Assay for IL-1 Activity

The activity of IL-1 was assayed using augmentation of the thymocyte proliferative response to a submitogenic dose of phytohemagglutinin (PHA, the comitogenic effect) as the indicator, according to Mizel [18]. Thymocytes (10^6 cells in 100 μl) from 4–7-week-old LPS nonresponding C3H/HeJ mice were cocultured with different dilutions of the sample (in 50 μl) and 2 μg/ml of PHA (in 50 μl) in microtiter plate wells. Each assay was carried out in triplicate. In the complete medium human serum was replaced with fetal bovine serum (Flow Laboratories). After 3 days the cultures were labeled with [^{3}H]thymidine (1 μCi/well) for 6 h and harvested using an automatic cell harvester. The dried filters were counted in a liquid scintillation counter (Wallac, Turku, Finland). The data are expressed as the mean of triplicate wells. The standard error of the mean was less than 10%. In each experiment the cells were derived from a single donor, and the samples were assayed on the same day. In each assay an internal control (LPS-induced monocyte culture supernatant) was tested simultaneously.

RNA Isolation and Analysis

Total cellular RNA was isolated by the guanidium isothiocyanate method with cesium chloride modification [19,20] and quantitated spectrophotometrically. Samples were size-fractionated on 0.8% formaldehyde-agarose gels, transferred to a nylon membrane (Pall, Glen Cove, NY), dried, and baked at 80°C under vacuum. The hybridization probes were for IL-1β, a synthetic 44-mer (a kind gift from Dr. Imre Cserpan, University of Stockholm, Sweden; for β-actin, a 27-mer oligonucleotide (Clontech Laboratories, Inc., Palo Alto, CA), and, for the glyseraldehyde phosphate dehydrogenase (GAPDH), the rat full-length cDNA insert from the pRGAPDH-13 [21]. For hybridizations with oligonucletide probes, filters were prehybridized at 42°C in solution containing 5 × SSC (750 mM NaCl, 75 mM sodium citrate), 20 mM sodium phosphate (pH 7.0), 10 × Denhardt's solution, 7% SDS, and 100 μg/ml heat-denatured salmon sperm DNA and hybridized overnight at 42°C in the same solution containing ^{32}P-labeled IL-1β or β-actin probes, which were labeled with ^{32}P-ATP using the T4-polynucleotide kinase. Filters were washed twice in 3 × SSC, 10 mM sodium phosphate (pH 7.0), 10 × Denhardt's solution and 5% SDS, and twice in 1 × SSC and 1% SDS at 60°C. For hybridization with GAPDH, prehybridization was

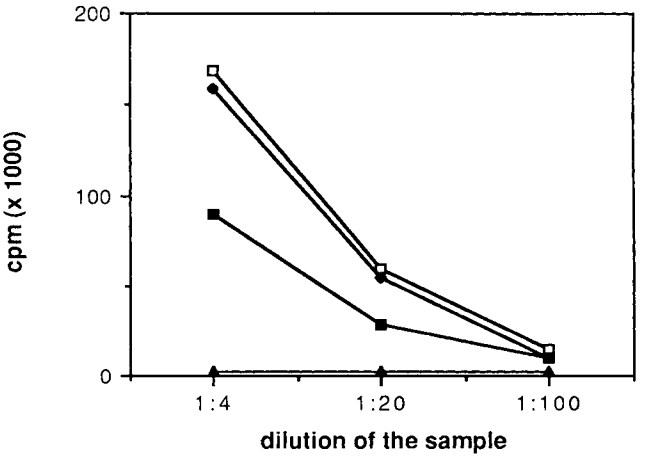

Fig. 1. IL-1 activity in the monocyte culture supernatants. Cultures were treated with 1 μg/ml of LPS (□) alone or with LPS plus TGFβ (▲, 10 ng/ml, ■, 1 ng/ml, ♦, 0.1 ng/ml), and the supernatants were collected after 24 h and tested in the thymocyte comitogenic assay at the dilutions indicated.

performed in solution containing 50% formamide, 1 × Denhardt's solution, 3 × SSC, 50 mM Hepes, and 150 μg/ml salmon sperm DNA, and the samples were hybridized overnight at 42°C in the same solution containing ^{32}P-GAPDH, labeled by nick-translation. Filters were washed in 1 × SSC and 0.1% SDS twice at room temperature and twice at 60°C. Filters were exposed to Kodak AR X-Omat films at −70°C, and autoradiograms were analyzed by laser densitometry.

RESULTS

Effect of TGFβ on IL-1 Production by Human Monocytes

To analyze the biological activity of the TGFβ used and to test its effect on IL-1 production, human monocytes were stimulated with LPS (1 μg/ml) alone or in combination with TGFβ (10, 1, or 0.1 ng/ml). After 24 h the culture supernatants were harvested and analyzed in the mouse thymocyte assay at the dilutions indicated (Fig. 1). It was found that LPS efficiently induced the secretion of IL-1 activity into the medium, but the presence of TGFβ in the culture supernatant had a clear suppressive effect. Supernatants from cultures stimulated with TGFβ alone did not contain any thymocyte-stimulating activity (data not shown).

These culture supernatants were fractionated by FPLC in a column in which TGFβ (mol. wt. 25,000) and IL-1 (mol. wt. 17,000) was separated; each fraction was then tested in the thymocyte comitogenic assay (Fig. 2). LPS-induced IL-1 activity was recovered as a single peak around 15,000, while the media derived from cultures stimulated with TGFβ alone did not contain any IL-1 activity. Culture media from cells stimulated with LPS plus 10 ng/ml or 1 ng/ml of TGFβ contained a clear peak of IL-1 activity, the magnitude of which was not, however, influenced by TGFβ. Taken together, these data indicate that TGFβ was biologically active (suppressing the proliferation of the test thymocytes), but when TGFβ and IL-1 biological activities were separated, it became evident that TGFβ did not have any effect on the IL-1 production of human monocytes.

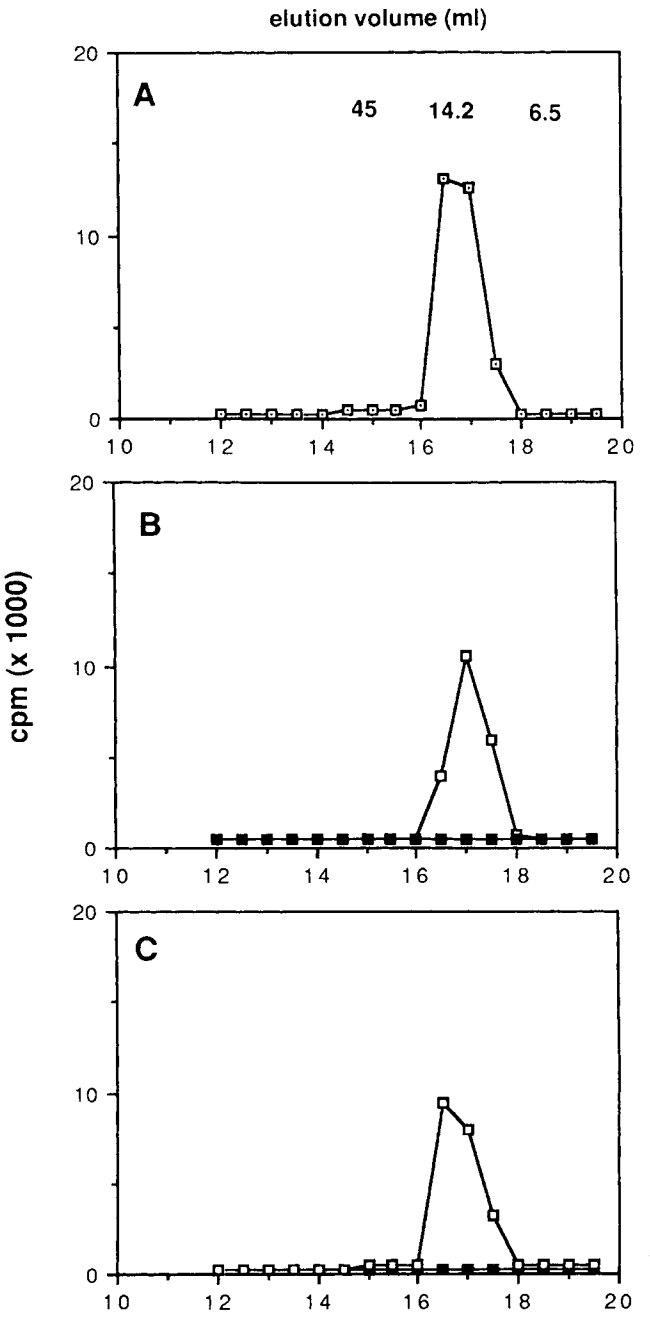

Fig. 2. IL-1 activity in the FPLC gel filtration column fractions of monocyte culture supernatants. Each column fraction was tested in the thymocyte comitogenic assay at 1:4 dilution. The calibration markers were ovalbumin (mol. wt. 45,000), α-lactalbumin (mol. wt. 14,200), and aprotinin (mol. wt. 6,500). **A:** Cultures treated with LPS (1 μg/ml) alone. **B:** Cultures treated with TGFβ (■, 10 ng/ml) alone or in combination with LPS (□). **C:** Cultures treated with TGFβ (■, 1 ng/ml) alone or in combination with LPS (□).

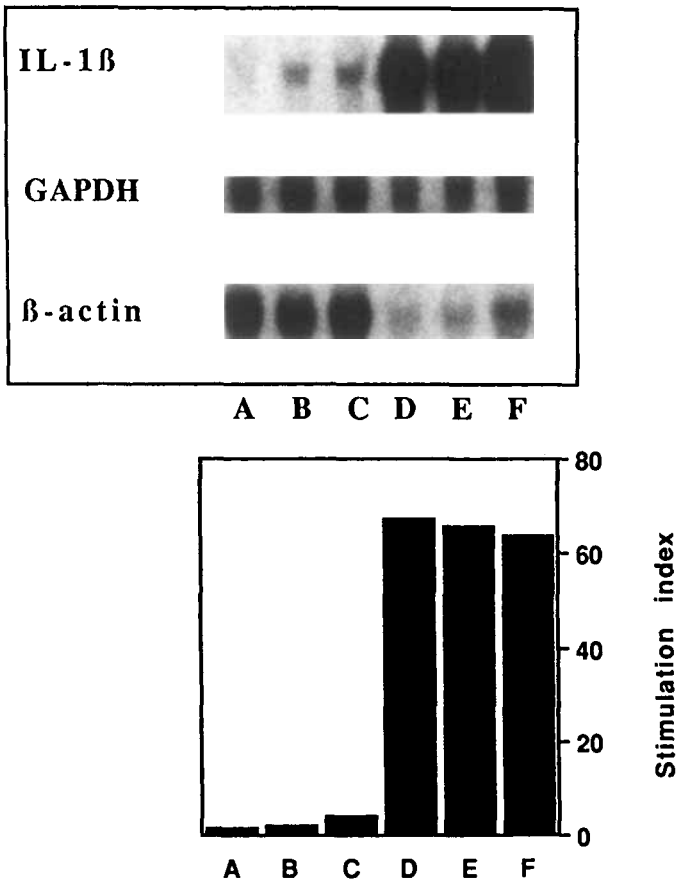

Fig. 3. Northern hybridization analysis of IL-1β mRNA in cultured human monocytes. Monocytes were cultured for 6 h in the presence of TGFβ or LPS before isolating total cellular RNA and analyzing the mRNA levels of IL-1β (1.6 kb), β-actin (2.0 kb), or GAPDH (1.3 kb). **Lane A:** Medium alone. **Lane B:** 1 ng/ml TGFβ, **Lane C:** 0.1 ng/ml TGFβ, **Lane D:** 1 μg/ml LPS. **Lane E:** 1 μg/ml LPS plus 1 ng/ml TGFβ. **Lane F:** 1 μg/ml LPS plus 0.1 ng/ml TGFβ. Lower panel shows the relative expression of IL-1β in the same groups (as determined by laser densitometric scanning after standardizing the mRNA amounts according to the expression of a constant gene, GAPHD).

Northern Hybridization Analysis of IL-1β mRNA in Cultured Human Monocytes

To observe the effects of TGFβ on the IL-1β mRNA level, total cellular RNA was isolated from monocytes after culturing them for 6 h with TGF (1 ng/ml or 0.1 ng/ml) alone or in combination with 1 μg/ml LPS. As shown in Figure 3, accumulation of IL-1β mRNA was not noticed after exposure to TGFβ. Also, TGFβ had no effect on LPS-induced accumulation of IL-1β mRNA. When the IL-1β mRNA was quantitated by using laser densitometry (standardizing the mRNA amounts according to the expression of constant probes, β-actin and GAPDH), it was obvious that TGFβ-induced IL-1β mRNA was at the background level.

As it is possible that adherence may modify the monocyte functions, we used

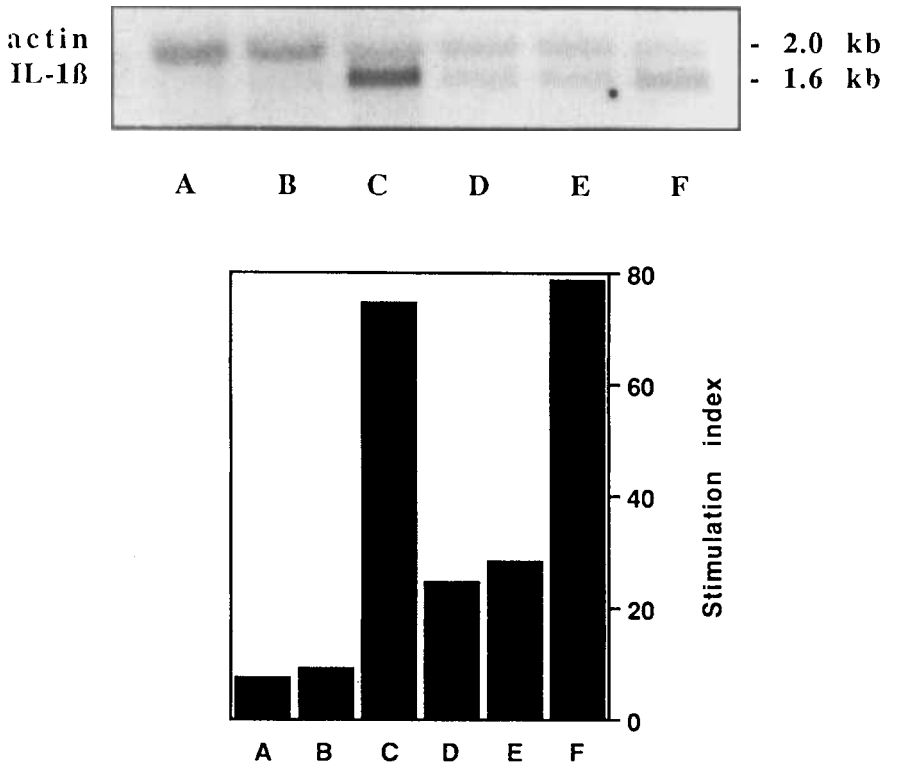

Fig. 4. Northern hybridization analysis of IL-1β mRNA in cultured human monocytes: comparison of the effects on adherence or Percoll gradient-purified monocytes. The monocytes were purified as described in Materials and Methods and cultured for 6 h in the presence of TGFβ or LPS before isolating total cellular RNA and analyzing the mRNA levels of IL-1β (1.6 kb) and β-actin (2.0 kb). **Lane A:** Medium alone. **Lane B:** 10 ng/ml TGFβ. **Lane C:** 1 μg/ml LPS. **Lane D:** Medium alone. **Lane E:** 10 ng/ml TGFβ. **Lane F:** 1 μg/ml LPS. Lanes A–C, adherence, lanes D–F, Percoll gradient-purified monocytes. The lower panel shows the relative expression of IL-1β in the same groups (as determined by laser densitometric scanning after standardizing the mRNA amounts according to the expression of a constant gene, β-actin).

discontinuous Percoll gradient for the monocyte preparation. Even a high dose of TGFβ (10 ng/ml) failed to enhance the levels of IL-1β mRNA levels (Fig. 4.). In addition, it was found that the background IL-1β mRNA expression was notably higher in the density-purified monocytes.

DISCUSSION

In the present study we did not find any evidence to support the concept that TGFβ would regulate IL-1 production in human monocytes. Specific IL-1β RNA in TGFβ-treated cultured human monocytes was at the level of nonstimulated cells, and, in addition, TGFβ did not have any effect on the IL-1β mRNA levels induced by LPS. TGFβ is usually active at concentrations around 4–40 pM (0.1–1 ng/ml), except in chemotaxis assays, in which considerably lower concentrations are sufficient [15,22]. Even at 10-ng/ml concentrations of TGFβ, no effects on IL-1 were observed.

The measurements of IL-1 bioactivities were fully in accordance with the RNA data. TGFβ was biologically active (suppressing the proliferation of thymocytes), and therefore it was necessary to separate the IL-1 and TGFβ activities by gel filtration to analyze the effect of TGFβ on IL-1 production. The findings showing that TGFβ did not have any effect on the production of biologically active IL-1 also exclude the possibility that TGFβ would induce only IL-1α. (RNA levels were measured by using a IL-1β-specific probe.)

Both TGFβ and IL-1 are able to induce PAI-1 in cultured fibroblastic and endothelial cells [10,23,24]. Our data do not support the possibility that TGFβ-mediated modulation of PA activity would be mediated via the induction of IL-1.

Our results contradict those of Wahl et al. [15]. They demonstrated that TGFβ induced levels of IL-1β mRNA as high as LPS in human monocytes. There is no obvious reason for this discrepancy. However, LPS is known to induce IL-1 production at very low concentrations, and sometimes even 10 pg/ml of LPS is sufficient to induce a measurable response [25]. Such LPS concentrations can often be present in standard tissue culture reagents.

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