Induction of the Interleukin 1 receptor antagonist protein by Transforming Growth Factor-β

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Induction of the interleukin 1 receptor antagonist protein by transforming growth factor-β*

Transforming growth factor-β1 (TGF-β1) mediates many immunosuppressive effects on immune cells and can inhibit the production of tumor necrosis factor and interleukin 1 (IL 1). However, TGF-β1 can stimulate the production of IL 6 and platelet-derived growth factor, indicating that TGF-β1 initiates complex effects on the production of cytokines. In this report we show that treatment of peripheral blood monocytes with TGF-β1 leads to the induction of a recently described IL 1 receptor antagonist protein (IRAP). The effect of TGF-β1 was both dose and time dependent. TGF-β1 induced *de novo* synthesis of IRAP, as Northern blotting experiments indicated a rapid and transient induction of the mRNA encoding IRAP. The induction of IRAP suggests a potential mechanism by which some of the inhibitory effects of TGF-β1 are mediated.

1 Introduction

Transforming growth factor-β1 (TGF-β1) mediates a number of inhibitory effects on immune cells, with an overall immunosuppressive action which suggests that it may possess immunosuppressive properties [1]. Thus, TGF-β1 is a potent inhibitor of lymphocyte growth and differentiation, blocking lymphocyte and thymocyte proliferation [2, 3], CTL and LAK cell generation [4, 5], and NK activity [6]. TGF-β1 has also been shown to inhibit B cell proliferation and IgG production [7]. In addition to its effects on lymphoid cells, TGF-β1 has been shown to block H_2O_2 production by MΦ [8].

Many of the effects of TGF-β1 on immune cell effector function could be indirect, by influencing the production of or responsiveness to stimulatory cytokines. We and others have shown that TGF-β1 has complex effects on the production of cytokines and their receptors. Thus, TGF-β1 blocked TNF and IL 1 production in response to bacterial LPS, but not to other stimuli such as PMA [9]. In addition, TGF-β1 blocked PHA-induced IFN-γ production by T lymphocytes, as well as causing inhibition of IL 2 and IFN-α receptor expression [2, 6]. In contrast to the inhibition of IL 1, TNF [9] and IFN-γ production [10], TGF-β1 can induce several cytokines including IL 6 [11] and platelet-derived growth factor (PDGF) [12], the latter cytokine appears to account for the mitogenic effects of TGF-β1 on fibroblast cell lines [12].

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Abbreviations: **IRAP:** IL 1R antagonist protein **PBM:** Peripheral blood monocyte **TGF:** Transforming growth factor **CM:** Conditioned medium **PDGF:** Platelet-derived growth factor

Recent data from several laboratories [13–15] has led to the isolation and cloning of an IL 1R-binding protein which has antagonistic properties to IL 1. This recombinant molecule, termed IL 1R antagonist protein (IRAP), has a molecular mass of 18–23 kDa and shows significant homology to IL 1α and IL 1β. An IL 1 antagonist (found to be equivalent to IRAP) with a molecular mass of 22–25 kDa has been shown to be produced by monocytes in response to differentiation signals such as adherent IgG and GM-CSF [13, 16].

In this study we demonstrate the ability of TGF-β1 to induce secretion of IRAP by peripheral blood monocytes (PBM). We suggest that one mechanism by which TGF-β1 modulates IL 1 effects and consequently the immune functions is through the induction of IRAP.

2 Materials and methods

2.1 Materials

PBMC were isolated from plateletpheresis residues from healthy donors (provided by the North London Blood Transfusion Service, Edgware, GB), on Ficoll-Hypaque gradients (Lymphoprep, Nyegaard, Norway). PBM populations were enriched by adherence to plastic as previously described [17]. Cells were maintained, and stimulated in complete medium which consisted of RPMI 1640, supplemented with 10% (v/v) FCS and 2 mM L-glutamine (Gibco, Paisley, Scotland). Human rTGF-β1 (LPS content < 0.125 EU/mg) was provided by Dr. M. Palladino (Genentech Inc., South San Francisco, CA).

2.2 IL 1 radioreceptor assay

Human rIL 1α (kindly provided by Peter Lomedico, Hoffmann-La Roche, Nutley, NJ) was iodinated to high sp. act. (30–45 μCi/μg = 1.11–1.67 MBq/μg), with >70% retention of biological activity, using the iodogen method as previously described [18]. For binding assays 2×10^6 cells (EL4 or 70Z/3) were washed in binding buffer (RPMI 1640 plus 10% FCS) and then preincubated with various dilutions of TGF-β1 conditioned medium (CM) or rIL 1 for 30 min at room temperature. ^{125}I-labeled IL 1α (1 nM) was then added to give a final volume of 200 μl and the samples

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were kept at 4 °C for 2 h on a shaking platform. Samples were then centrifuged through 20% sucrose to remove nonspecifically bound ligand and counted on a gamma counter. Nonspecific binding was determined in the presence of a 1000-fold molar excess of unlabeled IL 1α. Data are presented as percentage inhibition of binding, and are representative of experiments performed using three separate donors and three independently labeled batches of IL 1α.

2.3 Northern blotting

Total cellular RNA was prepared from enriched PBM by guanidinium isothiocyanate lysis and cesium chloride gradient ultracentrifugation, electrophoresed through 1% agarose gels containing 6% formaldehyde, as previously described [9]. Following electrophoresis, RNA was transferred onto nitrocellulose by capillary blotting. Filters were prehybridized in a solution containing 50% formamide, 5 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 7.5 × Denhardt's solution (1 × Denhardt's = 0.05% Ficoll 400, 0.05% polyvinylpyrollidone, 0.05% BSA), 50 mM sodium phosphate buffer (pH 6.6), 0.5 mg/ml denatured salmon sperm DNA at 42 °C for 42 °C for 4-16h. An IRAP cDNA probe was generated by performing the polymerase chain reaction on cDNA reverse transcribed from total RNA extracted from PBM treated with 10 ng/ml TGF-β1 for 9 h. The sequences of the primers used were derived from IRAP nucleotide sequence [14]. The 5′ oligonucleotide was 5′-CGCGCGAATTCCGGGCTGCAGTCACAGAATGG-3′ and the 3′ oligonucleotide was 5′-CGCGGGATCCATG-CAAGAATGGGAACAGGC-3′, respectively. Identity with IRAP cDNA was confirmed both by the size of the PCR product and by DNA sequence analysis. IRAP cDNA was gel purified and labeled by random oligo-priming using [α-^{32}P]dCTP (Amersham, Aylesbury, GB), denatured and then hybridized to filters overnight at 42 °C. Filters were washed twice in 2 × SSC, 0.1% SDS at room temperature, and twice in 0.2 × SSC, 0.1% SDS at 55 °C and exposed to Fuji X-ray film at −70 °C with intensifying screens.

2.4 Protein purification and identity

IRAP protein was purified from TGF-β1-CM by immunoprecipitation with antibodies directed against the IRAP protein followed by SDS-PAGE electrophoresis and Western blotting. Monocytes were enriched from peripheral blood as described above, and stimulated for 24 h in the presence or absence of TGF-β1 (10 ng/ml). SN were concentrated 3–4-fold by Amicon filtration (Amicon, Gloucester, GB), incubated with mAb directed to IRAP protein (I-4, I-10, I-12, I-15) (Upjohn, Kalamazoo, MI) and precipitated with rabbit anti-mouse Ig-coated Sepharose beads (Bio-Rad, Hemel Hempstead, GB). The beads were washed by applying a 15% sucrose gradient, centrifuged at 5000 × g for 10 min and frozen in a dry ice/ethanol bath. The precipitate was dissolved in 2 × SDS-PAGE sample buffer without reducing agent and separated on a 15% SDS-PAGE gel according to the method of Laemmli [19]. Recombinant IRAP protein (10–1000 ng/ml (Upjohn) was separated on the gel as a positive control. Proteins were transferred onto nitrocellulose and blotted with a fifth anti-IRAP mAb (I-5) (Upjohn). Proteins were detected with biotinylated goat anti-mouse Ig (Southern Biotechnology, Birmingham, AL) streptavidin/biotin horseradish peroxidase (Amersham) and visualized with an enhanced chemiluminescence Western blotting system detected by Hyperfilm-ECL (Amersham).

3 Results

TGF-β1-CM was generated by culturing PBM for 24 h with 10 ng/ml TGF-β1 and tested for its ability to compete with radiolabeled rIL 1α for binding to murine EL4 cells, which express the 80-kDa IL 1R [20]. TGF-β1 CM was a potent inhibitor of IL 1 binding to EL4 cells (Fig. 1a). This activity is not due to IL 1 since TGF-β1-CM does not contain immunoreactive or bioactive IL 1α or IL 1β [9]. The lack of IL 1 in these SN was confirmed by their inability to compete with radiolabeled IL 1α for binding to 70Z/3 cells (Fig. 1b). These cells express a form of the IL 1R which binds both IL 1α and IL 1β with high affinity, but is clearly a distinct gene product from that found EL4 cells [21, 22]. These properties are consistent with the presence of the recently cloned IRAP protein [13–15].

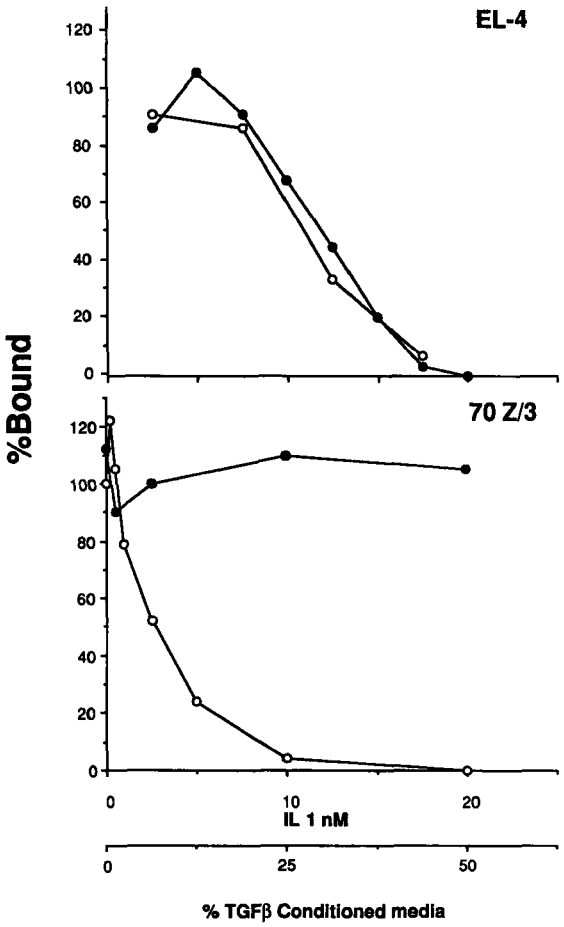

Figure 1. TGF-β1-CM inhibits IL 1 binding to EL4 but not 70Z/3 cells. PBM were cultured in serum-free RPMI for 24 h in the presence of TGF-β1 (10 ng/ml). SN were collected and assayed for their ability to block the binding of ^{125}I-labeled IL 1α to EL4 or 70Z/3 cells as described in Sect. 2.2. TGF-β1-CM (●) inhibited IL 1α binding to EL4 but not 70Z/3 cells. IL 1α (○) inhibition of IL 1 to EL4 and 70Z/3 cells served as a control.

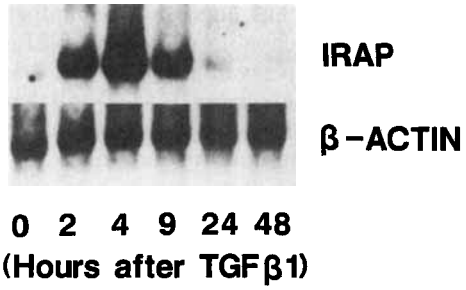

Figure 2. Northern analysis of IRAP induction by TGF-β1. PBM were cultured in 10 ml culture dishes at 1×10^6 cells/ml in RPMI 1640 containing 10% FCS and 10 ng/ml TGF-β1. RNA was harvested at the indicated times and 20 μg of total cellular RNA separated on a denaturing agarose gel, transferred to a nitrocellulose filter, and hybridized with an IRAP probe (top panel). Filters were stripped and rehybridized with a cell cycle invariant probe to actin (bottom panel).

In order to establish that TGF-β1 was indeed inducing IRAP we extracted RNA from PBM treated for various periods with human rTGF-β1 and performed Northern blots with an IRAP-specific probe. TGF-β1 transiently induced IRAP mRNA, the levels of which peaked about 4 h after TGF-β1 addition and thereafter gradually declined (Fig. 2). Levels of actin RNA, which served as a control, were unchanged by treatment with TGF-β1. We used the EL4 radioreceptor assay to establish a dose response for the induction of the IRAP protein. PBM were incubated for 24 h with various doses of TGF-β1 and CM was collected and assayed for IRAP. TGF-β1 induced a dose-dependent increase in IRAP activity in the CM (Fig. 3a). The kinetics of the induction of IRAP activity by TGF-β1 were then established by incubating PBM for various lengths of time in the presence of 10 ng/ml TGF-β1. TGF-β1 elicited an increase in IRAP production which was maximal by 12 h (Fig. 3b) whereafter IRAP levels began to decline. In contrast, detectable IRAP activity was not found in control SN until after 48 h of culture and these were significantly lower than those present in TGF-β1 CM (Fig. 3b).

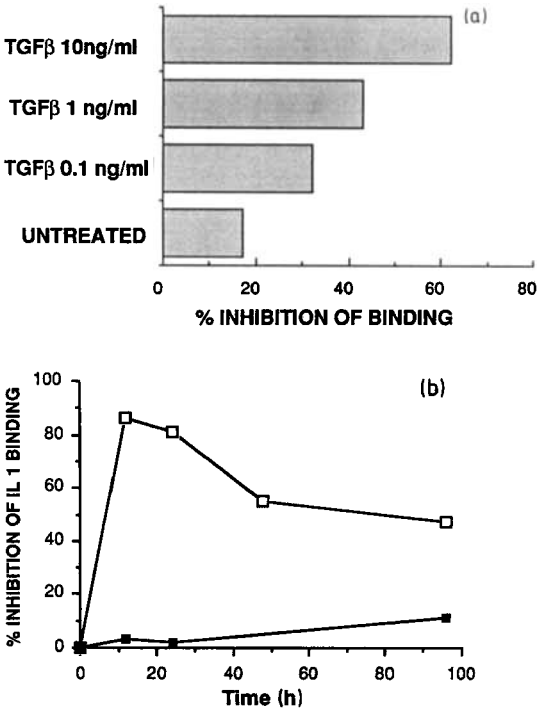

Figure 3. (a) Dose-response curve for TGF-β1 induction of IRAP. PBM at 1×10^6 cells/ml in serum-free RPMI 1640 were cultured with TGF-β1 (0–10 ng/ml), CM was collected after 24 h and assayed for ability to inhibit binding of ^{125}I-labeled IL 1α to EL4 cells as described in Sect. 2.2. (b) Kinetics of IRAP induction by TGF-β1. Freshly separated PBM were cultured for varying lengths of time at 1×10^6 cells/ml in RPMI 1640 and 10 ng/ml TGF-β1. CM (□) was collected and assayed for ability to inhibit binding of ^{125}I-labeled IL 1α to EL4 cells at the times indicated. Control cultures (■) consisted of PBM which were cultured for the times indicated in the absence of TGF-β1.

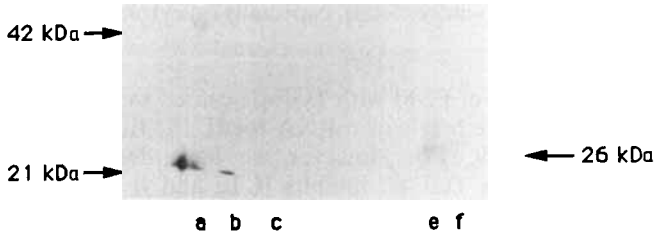

Figure 4. Biochemical characterization of IL 1 inhibitor activity. Monocyte-enriched PBM were cultured for 24 h in serum-free medium in the presence of TGF-β1 as described in the text. Monocytes cultured for an equivalent period of time in the absence of any stimulus served as a control. SN were precipitated with a cocktail of mAb directed against IRAP (as described in the text), separated by SDS-PAGE and Western blotted with a further anti-IRAP mAb. Recombinant IRAP protein lane a (1000 ng/ml), b (100 ng/ml), c (10 ng/ml) had a molecular mass of 21 kDa (42 kDa dimeric). TGF-β1-CM (lane e) contained protein with a molecular mass of 26 kDa. Lane f contained the monocyte control culture SN.

To confirm that the activity detected in TGF-β1-treated SN was indeed IRAP protein, TGF-β1-CM was precipitated and blotted with mAb directed against the IRAP protein. Based on molecular weight markers the precipitated complex was found to have a molecular weight of approximately 25 kDa (Fig. 4) compared with that reported for IL 1 antagonists of 22–25 kDa partially purified from immune complex or GM-CSF-stimulated monocytes [14, 16]. The difference between the recombinant IRAP (21 kDa) and TGF-β1-induced IRAP (26 kDa) observed in Fig. 4 is presumably due to differences in glycosylation.

4 Discussion

TGF-β1 has a multitude of effects on the immune and hemopoietic systems. The net effect is immunosuppressive and inhibition of the growth of T cells in response to IL 2, diminution of cytotoxicity and antibody formation have been extensively described [2, 4, 7]. This has led to proposals that TGF-β1 may be useful in regulating excessive immune responses, as in autoimmunity. However, the effects of TGF-β1 are not solely immunosuppressive. Thus, TGF-β1 enhances IgA production in the mouse [23], and we have shown that it induces the production of IL 6 protein [11]. Furthermore, the effect of TGF-β1 on immune actions is immediately noticeable if added prior to the immune stimulus; however, there is considerably less or no effect of TGF-β1 after the immune stimulus [9]. For these reasons we have investigated the mechanism of TGF-β1 action on

immune cells in more detail, especially on cytokine synthesis.

The treatment of PBM with TGF-β1 causes an increase in the steady state levels of mRNA for IL 1α, IL 1β, TNF-α and IL 6 [3, 9, 11]. However, we have demonstrated previously that TGF-β1 inhibits IL 1α and IL 1β protein production at the translational level [9] but that IL 6 is secreted in response to TGF-β1 [11]. This suggests that the translational blockade does not apply to all cytokines.

We found that the TGF-β1-conditioned monocyte medium inhibited binding of IL 1α only to EL4 cells and not to 70Z/3 cells. These mouse cells express different IL 1R, both of which are capable of binding IL 1α and IL 1β, but only the EL4 receptor can bind IRAP [13]. This suggested that TGF-β1-CM contained IRAP. Consistent with this we have never detected bioactive or immunoreactive IL 1 in TGF-β1-CM or cell lysates from TGF-β1-treated cells [9].

Thus the induction of IRAP by TGF-β1 was investigated and found to be dependent on the dose of TGF-β1 used. The kinetics of IRAP mRNA production in response to 10 ng/ml TGF-β1 were rapid, with peak levels attained within 12 h of TGF-β1 treatment. The mechanism of induction of IRAP by TGF-β1 involves an increase in IRAP mRNA levels, which could be due to increased transcription and/or mRNA stability.

Despite excitement about the potential clinical usefulness of IRAP, little has been reported about its physiology. Other inducers of IRAP have been described, including adherent IgG [13], and the cytokines GM-CSF and IL 3 [16, 24]. All of these agents induce the differentiation of monocytes into MΦ, but other differentiation-inducing agents such as IL 1, IL 4, TNF-α, α_1-acid glycoprotein and vitamin D3 do not appear to induce IRAP [25]. To our knowledge TGF-β1 has little effect on monocyte differentiation, and thus may act in a way distinct from these previously described inducers.

The results presented here may explain a discrepancy in the literature on TGF-β1 effects on IL 1 protein production. It was reported that TGF-β1 induced IL 1 mRNA and protein in human monocytes [3]. The evidence for the IL 1 protein in TGF-β1-CM was based on bioactivity in the fibroblast proliferation assay and the thymocyte assay (after unmasking IL 1 activity with anti-TGF-β1 antiserum), and upon inhibition of IL 1 binding in the IL 1R binding assay [3]. These results are now reconcilable. TGF-β1-induced PDGF production could account for fibroblast proliferation [12], IL 1 activity in the thymocyte assay could be due to the presence of IL 6 [11] and IRAP production (as described in this report) for the inhibition of IL 1R binding activity.

The effect of TGF-β1 on the IL 1 response is thus very complex. TGF-β1 induces IL 1 mRNA but not protein, TGF-β1 can inhibit the production of IL 1 induced by LPS or TNF but not PMA [9]. In a recent publication it was shown that TGF-β1 inhibited both constitutive and induced expression of IL 1R on murine lymphoid and myeloid progenitor lines [26]. Here we show the effect of TGF-β1 on generating an IL 1R antagonist (IRAP), thus TGF-β1 seems able to modulate both synthesis and response to IL 1. IRAP seems to be an important part of a local cytokine homeostatic network. Whether it also has important regulatory effects at a distance making it capable of being a useful therapeutic modality remains to be evaluated. The fact that it needs to be at marked molar excess over IL 1 [27] and that it acts on only one of the two IL 1R (in mouse) suggests that its major role may be in local immune regulation.

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