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# Interleukin 1 is an autocrine regulator of human endothelial cell growth

(endothelium/cytokines/cell proliferation)

FEDERICO COZZOLINO*†, MARIA TORCIA*, DONATELLA ALDINUCCI*, MARINA ZICHE‡, FABIO ALMERIGOGNA§, DANIELE BANI¶, AND DAVID M. STERN||

*Istituto di Clinica Medica IV, ‡Dipartimento di Farmacologia, §Istituto di Clinica Medica III, and ¶Istituto di Istologia, Università di Firenze, I-50139 Florence, Italy; and ||Department of Physiology and Cellular Biophysics, College of Physicians and Surgeons, Columbia University, New York, NY 10032

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ABSTRACT **Proliferation of endothelial cells is regulated through the autocrine production of growth factors and the expression of cognate surface receptors. In this study, we demonstrate that interleukin 1 (IL-1) is an inhibitor of endothelial growth *in vitro* and *in vivo*. IL-1 arrested growing, cultured endothelial cells in $G_1$ phase; inhibition of proliferation was dose dependent and occurred in parallel with occupancy of endothelial surface IL-1 receptors. In an angiogenesis model, IL-1 could inhibit fibroblast growth factor-induced vessel formation. The autocrine nature of the IL-1 effect on endothelial proliferation was demonstrated by the observation that occupancy of cell-surface receptors by endogenous IL-1 depressed cell growth. The potential significance of this finding was emphasized by the detection of IL-1 in the native endothelium of human umbilical veins. A mechanism by which IL-1 may exert its inhibitory effect on endothelial cell growth was suggested by studies showing that IL-1 decreased the expression of high-affinity fibroblast growth factor binding sites on endothelium. These results point to a potentially important role of IL-1 in regulating blood vessel growth and suggest that autocrine production of inhibitory factors may be a mechanism controlling proliferation of normal cells.**

Control of normal cell proliferation involves the interaction of factors both promoting and inhibiting cell growth (reviewed in ref. 1). In general, it is believed that such substances are conveyed from the extracellular environment; however, certain cell types, such as T lymphocytes or endothelial cells, have been shown to produce growth factors in an autocrine manner. In fact, secretion of interleukin (IL)-2 and IL-4 is a central event in T-cell proliferation, and regulated endogenous production of angiogenic factors promotes development of microvessels (2–4). It is known that T cells maintain tight control over this potentially explosive condition, at least in part by regulating the expression of growth factor receptors (5–7). An additional mechanism would include the production of growth inhibitors. These observations led us to consider that in endothelium, which constitutively produces a potent angiogenic agent, fibroblast growth factor (FGF), control of proliferation could occur through endogenous production of an inhibitor, which could act by controlling expression of the FGF receptor.

IL-1, a central mediator of the host response, is made by many cell types, including endothelium (8, 9). This cytokine induces changes in endothelial physiology, enabling these cells to participate actively in immune and inflammatory reactions (10–12). In several systems, IL-1 also acts as a growth-promoting substance, in addition to regulating synthesis of and responsiveness to other cytokines. In this study, we demonstrate that IL-1, a product of endothelial cells, interacts in an autocrine manner with high-affinity cell-surface receptors to inhibit endothelial growth. The mechanism of IL-1-induced suppression of endothelial cell proliferation is probably related to down-regulation of FGF receptors induced by this cytokine. These results contribute to the emerging complex picture of factors that regulate endothelial growth.

## MATERIALS AND METHODS

**Recombinant Cytokines and Antibodies.** Purified human recombinant (r) IL-1$\beta$ and -$\alpha$ were provided by A. Shaw (Glaxo, Geneva). Inactivation of the cytokines was accomplished by heating at 95°C for 20 min. r basic (b) FGF was provided by D. Rifkin (New York University, New York). IgG fractions of neutralizing anti-IL-1$\beta$ and anti-IL-1$\alpha$ sheep antisera were generous gifts of Stephen Poole (National Institute for Biological Standards and Control, Potters Bar, England), and preimmune sheep serum was obtained by Affi-Gel protein A (Bio-Rad) affinity chromatography. For experiments, each IgG fraction was used at a final concentration of 5 $\mu$g/ml. The latter concentration of anti-IL-1$\beta$ or anti-IL-1$\alpha$ IgG neutralized >15 ng of the respective cytokine, with no cross-reactivity, in the thymocyte costimulation assay (13). Monoclonal antibodies to IL-1$\beta$, also provided by A. Shaw, and to CD1 were purified from ascites by the methods described above.

**Cell Culture.** Endothelial cells were obtained from umbilical vein and were grown as described (14). For proliferation studies, cells (passages 3–5) were plated at a concentration of $2 \times 10^4$ per well in 24-well plates precoated with gelatin (0.2%) and incubated for 48 hr in the absence of exogenous growth factors. Next, medium was replaced with control medium containing no further additions or one of the following: rIL-1$\beta$, indomethacin (1 $\mu$g/ml), or lipopolysaccharide. During the final 12 hr of culture, cells were pulsed with 0.5 $\mu$Ci (1 Ci = 37 GBq) of [$^3$H]thymidine. DNA, precipitated with 10% (vol/vol) trichloroacetic acid, was collected on 0.2-$\mu$m filters and radioactivity was determined.

For cell cycle analysis, cells ($5 \times 10^5$ in 6-well plates) were cultured for 24 hr in medium without growth supplement before addition of rIL-1$\beta$ (1 ng/ml). After incubation for 18 hr, cells were washed and nuclei were obtained and stained as described (15). DNA content was analyzed on a FACStar flow cytometer (Becton Dickinson). Cell cycle compartments were evaluated by a nonlinear least-squares program for curve fitting. Fitted values for percentages of cells in $G_1$, S,

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Abbreviations: FGF, fibroblast growth factor; IL, interleukin; r, recombinant; b, basic.

†To whom reprint requests should be addressed at: Istituto di Clinica Medica IV, Università di Firenze, Viale Pieraccini 18, I-50139 Florence, Italy.

and $G_2$ plus M, respectively, were (for preconfluent cells) 42, 36, and 22. In the presence of IL-1, values were 67, 15, and 18.

**Radioiodination of Cytokines.** rIL-1$\alpha$ was radioiodinated by the method of Lowenthal and MacDonald (16) to a specific activity of 0.5 mCi/$\mu$g. rbFGF was labeled as described (17), and the specific activity was 0.15 mCi/$\mu$g. Both preparations of radioiodinated proteins ran as single bands on NaDodSO$_4$/polyacrylamide gel, with molecular masses of 17 and 18 kDa, respectively.

**Surface Binding of $^{125}$I-Labeled Cytokines.** For IL-1 studies, confluent cells ($1 \times 10^6$ in 6-well plates) were treated for 1 min at 4°C with medium buffered at pH 3.0 to elute endogenous ligand. The supernatant of this incubation is referred to as acidic eluate; the brief exposure of cultures to acidic buffer had no effect on cell viability, based on trypan blue exclusion. Then, cultures were washed in medium containing fetal calf serum (10%), and incubated for 2 hr at 4°C on an orbital shaker, in 1 ml of medium with different concentrations of $^{125}$I-labeled IL-1$\alpha$ alone or in the presence of excess unlabeled IL-1$\alpha$. Monolayers were then washed extensively and radioactivity was determined. In some experiments, acid-treated endothelial cells were incubated for 1 hr with the acidic eluate, derived from $\approx 10^6$ cells as described above, and, after washing, radioligand binding studies were performed.

For bFGF binding studies, endothelial cells ($5 \times 10^4$ in 24-well plates) were cultured in medium without exogenous growth factors for 24 hr and then exposed to IL-1$\beta$ for 16 hr. Binding experiments were then performed exactly as described (18).

**Biosynthetic Labeling and Immunoprecipitation Studies.** To assess IL-1 production, preconfluent endothelial cells ($1 \times 10^6$ cells per 25 cm$^2$) were metabolically labeled as described (19). Culture supernatants were collected, and monolayers were washed first with culture medium (neutral wash), then in glycine buffer (0.1 M, pH 2.8) (acidic eluate), and finally solubilized with Nonidet P-40 (1%). All of the samples were immunoprecipitated with anti-IL-1$\beta$ or anti-IL-1$\alpha$ IgG, or with preimmune sheep IgG, and analyzed in a NaDodSO$_4$/10% polyacrylamide gel, as described (20).

**Immunohistochemistry.** Fresh umbilical veins were isolated, fixed in formaldehyde, and embedded in paraffin. Sections (5 $\mu$m) were stained with anti-IL-1$\beta$ or with anti-CD1 monoclonal antibodies, according to the peroxidase-antiperoxidase method (21), using diaminobenzidine as chromogen, and counterstained with Mayer's hematoxylin.

**Angiogenesis Assay.** Slow-release pellets containing either rbFGF, rIL-1$\beta$, or both (each at 50 ng per pellet) were prepared by incorporating the test material into a casting solution of Elvax-40 (DuPont) in 10% methylene chloride. Pellets were then left to evaporate under vacuum at 4°C overnight. New Zealand White rabbits were anesthetized and a micropocket was produced in the avascular cornea as reported (22). All procedures were performed under sterile conditions.

## RESULTS AND DISCUSSION

In the course of studies on the effects of IL-1 on endothelial coagulation mechanisms, it became evident that IL-1-induced perturbations extended beyond alteration of cell-surface properties and included changes in cell growth. Exposure of cultured subconfluent endothelium to recombinant IL-1$\beta$ led to a dose-dependent decrease in their incorporation of [$^3$H]thymidine: suppression was maximal at 36 hr (Fig. 1*A*) and was dependent on the concentration of IL-1, being half-maximal at $\approx$2 pM (Fig. 1*B*). In each case, decreased [$^3$H]thymidine incorporation correlated with a decline in cell number. Abolition of the effect of IL-1 on

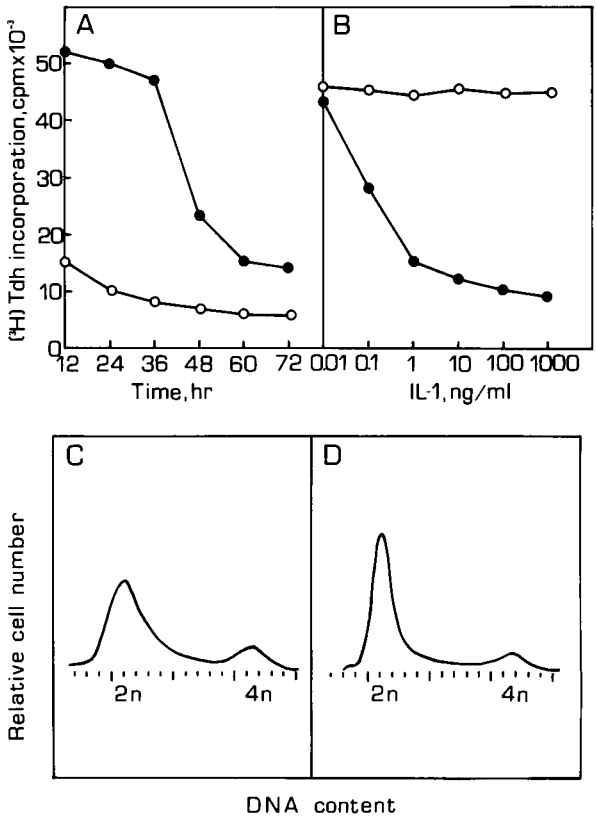


FIG. 1. Effect of IL-1 on endothelial cell proliferation. Cells were cultured in the absence (●) or presence (○) of rIL-1$\beta$ (1 ng/ml) for the indicated times (*A*), or with the indicated concentrations of rIL-1$\beta$ for 36 hr (*B*), in the presence (○) or absence (●) of neutralizing anti-IL-1$\beta$ antibodies. Results are expressed as mean [$^3$H]thymidine ([$^3$H]Tdh) incorporation of triplicate cultures; SD was <10%. Data from 1 representative experiment of 10 performed are shown. Distribution of cell cycle phases among preconfluent endothelial cells without (*C*) or with (*D*) rIL-1$\beta$ is shown. Representative distributions from three experiments are shown.

endothelial growth by monospecific neutralizing antibodies to IL-1 or by substitution of heat-inactivated IL-1 for the native molecule (Fig. 1*B*) confirmed that active IL-1 molecules were responsible for growth inhibition. The possible role of growth-inhibiting prostaglandins (23) was ruled out by experiments with indomethacin-treated endothelial cells (data not shown). Furthermore, flow microfluorimetry showed that IL-1 markedly reduces the percentage of cells in S phase from 35% (typical of an exponentially growing culture) to $\approx$10% (Fig. 1 *C* and *D*), suggesting arrest in $G_1$, as also occurs in endothelial cultures treated with transforming growth factor type $\beta$ (15). These findings are consistent with those reported by Norioka *et al.* (24), in which, however, IL-1 effects on endothelial cell growth required higher concentrations of cytokine.

In view of the growth-promoting properties of IL-1 in most of the systems so far studied, it was important to extend these findings with cultured cells to an *in vivo* angiogenesis model. Using the rabbit cornea assay, IL-1$\beta$ was shown to be a potent inhibitor of neoangiogenesis induced by recombinant bFGF (Fig. 2). In contrast, heat-inactivated IL-1 had no effect on the strong angiogenesis observed in response to bFGF. Control studies performed with IL-1 alone produced mild angiogenesis; however, this effect was more pronounced with heat-inactivated cytokine, suggesting that contaminants active in the assay were present in the recombinant preparation. These data indicate that IL-1 can block neovascularization in response to a potent angiogenic stimulus, such as bFGF, setting IL-1 apart from other cytokines described

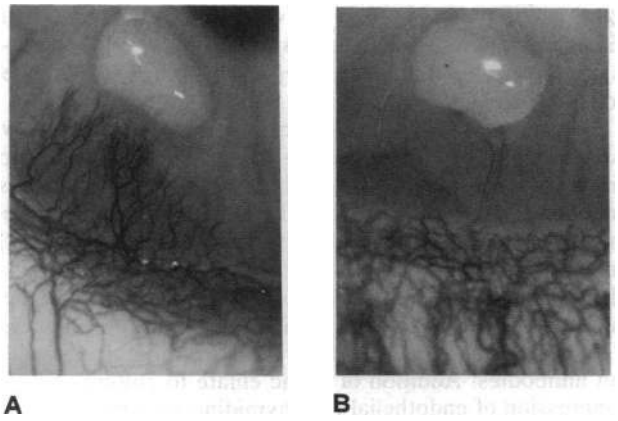


FIG. 2. Effect of IL-1 on bFGF-induced angiogenesis. In the same rabbit bFGF activity (*A*) was compared to that of bFGF and IL-1 (*B*). bFGF induced neovascularization in six of six implants and the vascular network persisted unmodified for 2 weeks. The angiogenic activity of implants bearing simultaneously bFGF and IL-1 appeared slowly progressing compared to bFGF alone, and after 1 week only three of six implants showed a positive score. At the end of the 2nd week of observation the newly formed vascular network appeared further reduced in terms of vessel number. When heat-inactivated IL-1 was tested with bFGF, a strong angiogenesis occurred.

so far (e.g., transforming growth factor type $\beta$ and tumor necrosis factor type $\alpha$), which enhance formation of blood vessels *in vivo*, while inhibiting endothelial growth *in vitro* (15, 25–27). Furthermore, this effect of IL-1 on endothelium is in contrast to its ability to induce proliferation of other cells, such as those from Kaposi sarcoma (19) or vascular smooth muscle (23).

To examine the mechanism of IL-1-mediated suppression of endothelial growth, we studied the relationship between this cytokine's effect on proliferation and occupancy of high-affinity cell-surface binding sites. Only lower-affinity endothelial binding sites for IL-1 with a $K_d$ of $\approx$0.7 nM have been hitherto demonstrated (28). Since endothelium has been shown to produce IL-1 *in vitro*, we considered that endogenous IL-1 was occupying the higher-affinity binding sites, allowing detection of only the lower-affinity binding sites. After elution of the cell surface with acidic buffer (a condition that dissociates many ligand–receptor interactions) to remove endogenous ligand, binding studies demonstrated high-affinity binding sites for $^{125}$I-labeled IL-1 with a $K_d$ of $\approx$4 pM, and 100 sites per cell (Fig. 3). Occupancy of these sites by IL-1 correlated closely with IL-1-induced inhibition of endothelial proliferation, suggesting a central role for these receptors in suppression of cell growth *in vitro*.

Consistent with the above findings, although endothelial cell culture supernatants had no IL-1 activity, acidic eluates of the cell surface did contain IL-1 activity (Table 1). In addition, material in this acidic eluate could prevent in a dose-dependent manner the binding of exogenous $^{125}$I-labeled IL-1 to endothelium, as did exogenous rIL-1, even after exposure to acidic conditions. After chromatography of the eluate over a column of immobilized anti-IL-1$\beta$ IgG, however, it no longer blocked $^{125}$I-labeled IL-1 binding, in parallel with removal of IL-1 activity (Table 1). The source of IL-1 activity in culture supernatants was probably the endothelium, since all sera were heat treated to destroy IL-1, and prolonged incubation (up to 24 hr) of cultures in serum-free medium failed to affect the presence of IL-1-like activity in the acidic wash.

These data suggested that endogenous IL-1 was present on the cell surface and could be eluted under acidic conditions. To demonstrate this directly, culture supernatants, eluates

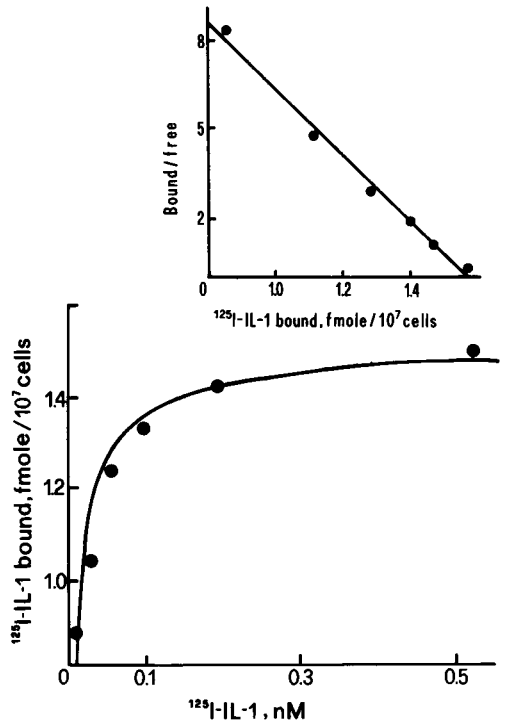


FIG. 3. IL-1 binding by endothelial cells. Saturation curve at equilibrium (*Lower*) and Scatchard representation (*Upper*) of the specific binding. Data shown are representative of three different experiments.

from intact cells (obtained under acidic and neutral conditions), and cell lysates from metabolically labeled endothelial cultures were immunoprecipitated with anti-IL-1$\beta$ antibodies (Fig. 4). The mature form of IL-1$\beta$, the band corresponding to a mass of 17 kDa, was specifically detected only in the acidic eluates from intact cells, whereas precursor IL-1$\beta$, the band corresponding to a mass of 33 kDa, was in cell lysates. No IL-1$\beta$ was detectable in culture supernatants or in the neutral wash of the cells. The same pattern was observed with anti-IL-1$\alpha$ antibodies but with less intense bands (data not shown). These results indicated that endothelial production of IL-1 is under tight control; no material is released into culture supernatants [consistent with the recent observation

Table 1. IL-1 activity of endothelial cell eluates and their effect on binding of radiolabeled IL-1$\alpha$

| Material added | IL-1 activity, cpm | Bound radioactivity, cpm |
|---|---|---|
| Medium alone | 1,856 | 756 |
| rIL-1$\alpha$ (1 nM) | 28,050 | 42 |
| Neutral eluate (1:2) | 2,123 | 803 |
| Acidic eluate | | |
| 1:2 | 8,405 | 61 |
| 1:4 | 5,643 | 237 |
| Absorbed acidic eluate (1:2) | 2,518 | 724 |

Neutral and acidic eluates were obtained from endothelial cells cultured at $10^6$ per well for 48 hr and tested, either absorbed or not on anti-IL-1$\beta$ IgG coupled to solid phase, for IL-1 activity and the capacity of inhibiting binding of $^{125}$I-labeled IL-1, at the indicated dilutions. IL-1 activity was assessed by the conventional thymocyte costimulation assay. Results are expressed as mean [$^3$H]thymidine incorporation of triplicate cultures. SD was always <15%. Endothelial monolayers ($10^6$ per well) were acid treated to remove endogenous IL-1, incubated or not with test material, and exposed to 0.1 nM $^{125}$I-labeled IL-1$\alpha$.

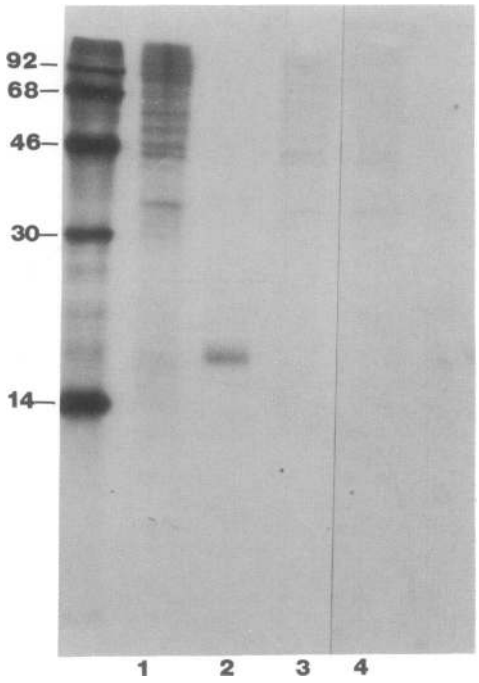


FIG. 4. IL-1 production by endothelial cells *in vitro*. (*A*) Immunoprecipitation analysis of IL-1$\beta$ in cell lysates (lane 1), supernatants from acid pH washing (lane 2), supernatants from neutral pH washing (lane 3), and culture supernatants (lane 4) obtained by preconfluent cells cultured with [$^{35}$S]methionine and [$^{35}$S]cysteine. Supernatants, neutral and acid pH washing fluids, and cell lysates were immunoprecipitated with monospecific anti-IL-1$\beta$ antibodies and analyzed by electrophoresis on a NaDodSO$_4$/10% polyacrylamide gel. Numbers on left are kDa.

of Ensoli *et al.* (19)], but IL-1 is tightly bound to the cell surface.

Since endothelial production of IL-1 has been studied mostly *in vitro* (29), it was important to obtain evidence that IL-1 was present in native endothelium *in vivo*. Immunoperoxidase staining (using monoclonal antibodies to IL-1$\beta$) on native endothelium *in situ* in umbilical veins showed IL-1 within the cells (Fig. 5); the intensity of staining was rather low, suggesting that only small amounts of antigen may be present. The presence of IL-1 in the umbilical endothelium is in general agreement with recent reports demonstrating IL-1 gene expression in several normal human and mouse tissues

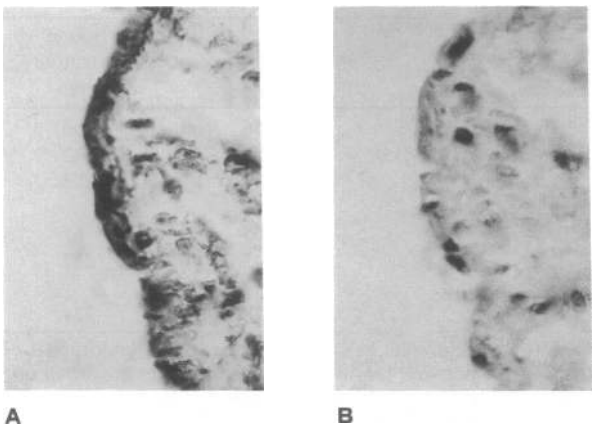


FIG. 5. Immunocytochemical detection of intracellular IL-1 in human umbilical vein sections stained with anti-IL-1$\beta$ monoclonal antibody (*A*). No staining is observed in a consecutive section when substituting the specific antibody with an irrelevant control antibody (anti-CD1) (*B*). (×640.)

(30, 31), although its levels of expression in vascular tissue may be quite low. These data support the concept that IL-1 is present in endothelium *in vivo* and may be produced constitutively.

Since endothelium both produces and responds to IL-1, we considered whether endogenous IL-1 could have an autocrine role in controlling endothelial cell growth. To test this, endothelial cells were eluted with acidic buffer and cultured in the presence or absence of neutralizing antibodies to IL-1$\beta$ and of acidic eluate prepared from a larger culture ($\approx 10^6$ cells) (Table 2). Endothelial cells subjected to acid elution and then cultured with anti-IL-1 antibodies demonstrated enhanced [$^3$H]thymidine incorporation, as compared to cultures without antibodies. Addition of acidic eluate to cultures caused suppression of endothelial [$^3$H]thymidine incorporation that was prevented by anti-IL-1 antibodies. Taken together, these data are consistent with the concept that IL-1 was produced by the cells and bound to the cell surface, where it functions as a negative autocrine regulator of endothelial growth. The ability of exogenous IL-1 to further suppress endothelial growth (Fig. 1) probably reflects incomplete occupancy of cell-surface receptors by endogenous IL-1, as might be expected for a system designed to be highly responsive to environmental stimuli.

After occupancy of the endothelial IL-1 receptor, events are set in motion that appear to inhibit cellular proliferation. Exogenous IL-1 added to endothelial cultures induces production of IL-6, which in turn modulates cell growth (11, 32); however, neutralizing anti-IL-6 antibodies do not revert growth inhibition (data not shown), indicating that most of the IL-1 activity is exerted through a direct pathway. In view of the inhibitory effect of IL-1 on FGF-stimulated angiogenesis *in vivo*, we considered whether the cytokine could interfere with expression of FGF receptors. Preincubation of endothelium with IL-1$\beta$ (1 ng /ml) resulted in decreased specific binding of $^{125}$I-labeled bFGF (Fig. 6). Analysis of the binding data indicated that this was due to a decrease in the number of high-affinity ($K_d$, $\approx$23 pM) binding sites for FGF, from $\approx$4000 per cell in the absence of IL-1 to $\approx$600 per cell in the presence of IL-1. Experiments with a range of IL-1 concentrations (0.01–10 ng/ml) demonstrated that the effect of IL-1 on the FGF binding sites was dose dependent. Consistent with results of these radioligand binding studies, IL-1 depressed bFGF-stimulated incorporation of [$^3$H]thymidine by cultured endothelium (Table 2) in a manner that paralleled the decrease in FGF binding sites. These data are consistent with the hypothesis that IL-1 can down-regulate expression of FGF receptors and suggest a possible mechanism by which IL-1 depresses endothelial growth.

Table 2. Effect of endogenous IL-1 and exogenous bFGF on endothelial cell proliferation

| | [$^3$H]Thymidine incorporation, cpm | | | |
|---|---|---|---|---|
| Stimulus | Medium | Acid eluate | rIL-1 (10 pg/ml) | rIL-1 (100 pg/ml) |
| None | 36,537 | 20,248 | 18,874 | 9,834 |
| Anti-IL-1 IgG | 65,746 | 56,438 | 58,438 | 55,345 |
| bFGF (10 ng/ml) | 72,675 | 55,438 | 48,341 | 10,437 |
| bFGF (1 ng/ml) | 52,246 | 38,343 | 35,446 | 9,874 |

Endothelial cells ($2 \times 10^4$) were acid treated, washed, and cultured for 48 hr in the presence or absence of the indicated reagents. Acid eluate was obtained from $10^6$ endothelial cells, neutralized by addition of NaOH, and used at a final dilution of 1:4. Data are expressed as mean [$^3$H]thymidine incorporation of triplicate cultures. SD was always <10%. The experiment was repeated three times with no significant variations in the results. IgG fractions of rabbit neutralizing anti-IL-1$\beta$ antiserum or of preimmune serum were used at a final concentration of 5 $\mu$g/ml.

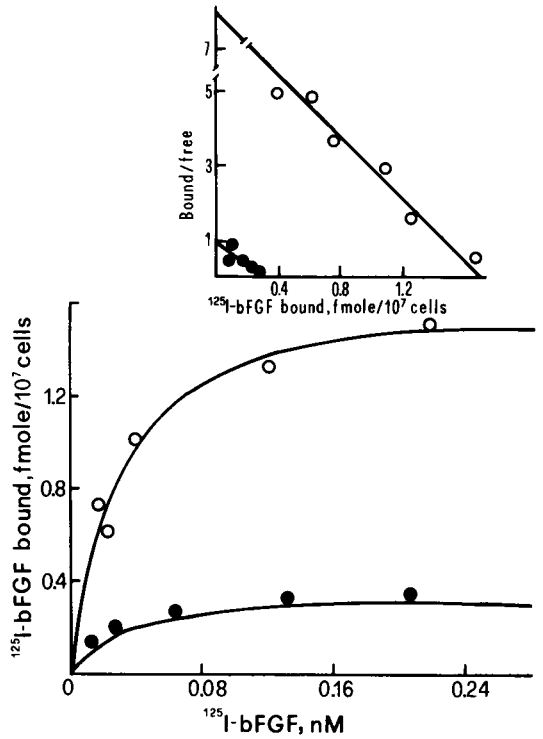


FIG. 6. Effect of IL-1 on bFGF binding to endothelial cells. Saturation curve at equilibrium (*Lower*) and Scatchard representation (*Upper*) of the specific binding of $^{125}$I-labeled bFGF to endothelial cells cultured in the absence (○) or presence (●) of rIL-1$\beta$. Data shown are representative of three different experiments.

Autocrine production of bFGF by endothelium has been reported recently (4). Our demonstration of autocrine production of IL-1 as a factor suppressing endothelial growth thus defines the endothelium as a self-regulating system, in which external control of growth may occur by altering expression of endogenous positive and negative mediators. This eventually results in an intensified or diminished input of signals delivered to the cell through the surface receptors; processing of such inputs governs the final proliferative or maturational choice of the cell. In this context, autocrine production of growth-inhibitory factors may reveal a common control mechanism of normal cell function. The ongoing production of IL-1 would limit the effects of the heparin-binding growth factors embedded in the extracellular matrix (33) through down-regulation of FGF receptor expression. *In vivo*, a preponderance of IL-1 would be expected during granuloma formation and within granulomata, loci notoriously avascular and subject to central necrosis. In addition, in wound repair major neoangiogenesis usually occurs during or after recession of the IL-1-producing macrophages has begun. The observation of IL-1-induced reduction in bFGF binding sites on endothelium suggests that a common feature of cytokine–growth factor interaction may be alterations in the expression of growth factor receptors, as also exemplified by transmodulation of epidermal growth factor receptors by tumor necrosis factor type $\alpha$ (34).

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