system [T. F. Donahue, R. S. Daves, G. Lucchini, G. R. Fink, *Cell* **32**, 89 (1983)]. Thus, 4% levels of expression, as noted by the β-gal assay, will increase to 16 to 20% of repressed levels, which are sufficient for growth. This property has been established for other *his4* alleles and their corresponding *his4-lacZ* constructions (*23*).

30. An AGG codon was introduced 28 nucleotides upstream of the AGG codon at the +1 initiator region by site-directed mutagenesis with the oligonucleotide 5′-TAATACAAGGAATTCCAAAATTT-3′. The methods used for constructing the mutation, the DNA sequencing strategy to confirm the construction, and the subsequent *his4* allele construction have been previously described (*6, 23*). As a result of this mutagenesis the natural sequence in the *HIS4* leader region between positions −28 and −21, AUAGUUUA, was changed to AGGAAUUC.
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Endothelial Adhesiveness for Blood Neutrophils Is Inhibited by Transforming Growth Factor–β

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Adhesion of blood cells to endothelial cells is an essential component of all inflammatory responses. The capacity of the endothelium to support adhesion of neutrophils is increased by cytokines such as tumor necrosis factor–α, interleukin-1, and endotoxin. Another cytokine, transforming growth factor–β (TGF-β), was a strong inhibitor of basalneutrophil adhesion and also decreased the adhesive response of endothelial cells to tumor necrosis factor–α (TNF-α). The ability of cells to respond to TGF-β was related to the duration of culture of endothelial cells after explantation from umbilical veins. TGF-β is likely to serve an anti-inflammatory role at sites of blood vessel injury undergoing active endothelial regeneration.

ATTACHMENT TO THE ENDOTHELIUM is essential in the movement of cells from the circulation into the site of an inflammatory reaction. The level of cell attachment is markedly increased by cytokines such as TNF and interleukin-1 (IL-1), which also increase the levels of some molecules on the endothelial surface involved in adhesive reactions (*1–3*).

Endothelial cells also elaborate an extracellular matrix composed of glycoproteins such as laminin (*4*), fibronectin (*5*), and collagen (*6*) involved in cell anchorage and migration. Recently, TGF-β was shown to increase the incorporation of fibronectin and collagen into the extracellular matrix of fibroblasts, epithelial, and endothelial cells (*7, 8*). Furthermore, TGF-β also inhibits endothelial cell proliferation in response to growth-promoting factors (*9*) and wounding (*10*), which suggests that TGF-β may be an important regulator of function at the endothelial surface. We therefore examined the effect of this molecule on adhesive interactions involving endothelial cells and neutrophils.

Primary cultures of human umbilical vein (HUVE) endothelial cells exposed to TGF-β for 24 hours showed a reduced capacity to support neutrophil binding (Fig. 1) that was dose-dependent but plateaued at maximum levels of inhibition with TGF-β (2 ng/ml). This dose response for inhibition of neutrophil attachment is consistent with reports for the effect of TGF-β on endothelial cell proliferation, locomotion, and angiogenesis (*8*). The response was blocked by a monospecific antibody against TGF-β (R&D Systems) and similar inhibition was demonstrated with recombinant (r) TGF-β (Genentech); the basal adherence of 0.149 ± 0.016 was reduced to 0.063 ± 0.002 by rTGF-β (2 ng/ml). This dose of TGF-β did not result in inhibition of endothelial cell proliferation; there was little or no change in the morphology or density of the endothelial monolayer as assessed by dye uptake and microscopy. Cell numbers after 24 hours of exposure to TGF-β (2 ng/ml) were $10.6 \pm 0.8 \times 10^3$ and without exposure to TGF-β were $9.6 \pm 0.8 \times 10^3$ (arithmetic mean ± SEM, $n = 5$) from an original 10^4 cells plated per well.

To test the effect of varying the time of addition of TGF-β on the inhibition of neutrophil attachment, endothelial cells were replated into microtiter wells, and treated for 3 to 24 hours with TGF-β (2 ng/ml) before assaying for neutrophil binding. Significant inhibition of adherence was seen after a 6-hour exposure; however, maximum inhibition was obtained when TGF-β was added at the initiation of the experiment and the endothelial cells were exposed for a full 24-hour period (Table 1). Again, no effect on endothelial cell growth was observed under these conditions.

TGF-β has been shown to decrease the number of high-affinity epidermal growth factor (EGF) receptors on a variety of endo-

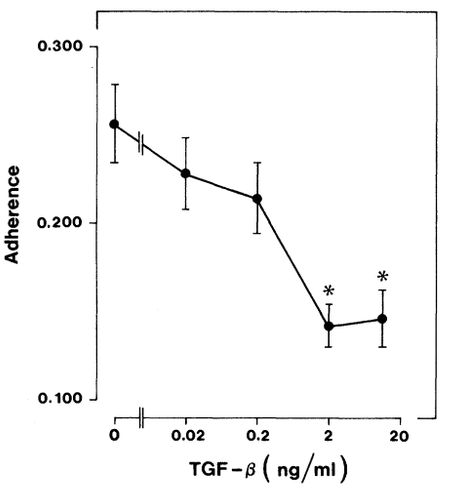

Fig. 1. The effect of TGF-β on adherence of neutrophils to endothelial monolayers. HUVEs obtained from collagenase treatment of umbilical cords were cultured for 3 to 7 days in 25 cm^2 flasks (Costar) in RPMI 1640 plus 20% fetal bovine serum (FBS) as described (*17*). Cells were then harvested by trypsinization (Flow) and plated into 6.41-mm^2 microtiter wells (NUNC) at 1.6×10^4 cells per well. TGF-β (97% pure isolate from human platelets, R&D Systems) was then added and after 24 hours the adherence of Ficoll-Hypaque–purified (*18*) venous blood neutrophils (mean purity 96%) was measured with the use of an assay based on the uptake of the viable stain Rose Bengal (*19*). Between 2 and 8×10^5 neutrophils in RPMI 1640 plus 5% FBS were added to wells of a 96-well flat-bottomed microtiter tray containing monolayers of HUVEs. After 30-min incubation at 37°C in 5% CO_2, the supernatant was removed and 100 μl of 0.25% Rose Bengal stain was added for 5 min at room temperature. Nonadherent cells were removed by two subsequent washes in medium; stain incorporated into the cells was released by the addition of 200 μl of ethanol:phosphate-buffered saline (1:1). After 45 min the wells were read in an enzyme-linked immunosorbent assay (ELISA) reader (Dynatech) at 570 nm. The level of adherence is given as the mean optical density reading at 570 nm (OD_{570}) of wells containing adhering cells and HUVEs minus the mean OD of wells containing HUVEs alone. On the average a tenfold change in OD_{570} represents a tenfold change in the numbers of cells adherent per square millimeter. Each point represents the arithmetic mean ± SEM of 9 to 15 determinations from four to five separate experiments. Points marked by an asterisk differ significantly from no added TGF-β ($P \leq 0.005$, two-tailed t test).

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thelial cell types and to prevent the EGF-mediated induction of several growth regulatory genes (*9*). This suggested that TGF-β might affect the adhesiveness of endothelial cells by altering the capacity of the cells to respond to other adhesion-promoting cytokines. HUVE monolayers exposed for 24 hours to TGF-β (2 ng/ml) were stimulated for 4 hours with the cytokine TNF-α, followed by assessment of neutrophil binding (Fig. 2). As we have previously shown (*1*) TNF-α stimulation of HUVEs increases neutrophil attachment in a dose-dependent fashion. Addition of TGF-β inhibited neutrophil binding to unstimulated HUVEs and also to TNF-α–stimulated HUVEs at all doses of TNF-α tested. Preincubation with TGF-β completely abolished the normal enhancement of adhesion caused by TNF-α.

Three types of TGF-β receptors have been found in bovine endothelial cells. The largest (280 kD, type III) receptor is only present on subconfluent cells, whereas the smaller receptors (85 kD and 72 kD, types I and II) are seen on cells from confluent cultures (*8*). Because the pattern of expression of these receptors varies with the stage of cell growth, we decided to test whether ligand binding to the TGF-β receptor on human endothelial cells may result in different functional effects when the cells were at different stages of cell growth. We therefore tested endothelial cells, grown for varying lengths of time, for their ability to respond to TGF-β as assessed by neutrophil attachment. HUVEs recently established in culture (3 to 6 days) showed responsiveness to TGF-β by a decreased level of neutrophil adherence. However, when subcultures were allowed to grow for an additional 3 to 8 days they no longer responded to TGF-β in this assay (Fig. 3). This change in TGF-β–responsive phenotype was not related to confluency of the HUVEs at the time of the adherence assay because the cells were always replated at the same densities. The phenomenon appeared to be associated with the age of the endothelial cells and may be related to the presence or absence of the high molecular weight receptors (*8*). Indeed, the triggering of the type III receptor in fibroblasts leads to the induction of the adhesion-promoting molecules fibronectin and collagen (*7, 11*) and to an increase in proteoglycan expression (*12*).

Our findings on adherence may be related to the expression on endothelial cells of a group of adhesion-promoting molecules such as ELAM-1 (endothelial-leukocyte adhesion molecule–1) (*13*) and ICAM-1 (intracellular adhesion molecule–1) (*14*), the expression of which are enhanced by TNF-α. TGF-β treatment of recently established and actively growing endothelial cells may inhibit adhesion by preventing the expression or up regulation of these molecules. Preliminary evidence suggests that TGF-β does down regulate the surface expression of ICAM-1 (*15*). The effect of TGF-β on adhesion thus appears to be highly selective, as (i) it promotes adhesion by elaborating extracellular matrix proteins such as fibronectin (7) and by increasing the expression of its receptor (7), but (ii) it prevents endothelial cells from exhibiting an adhesive phenotype for neutrophils and prevents the response to the proinflammatory cytokine, TNF-α.

In addition to enhancing endothelial cell adhesiveness, TNF also induces tissue factor and plasminogen activator inhibitor on endothelial cells (*16*) thus favoring a thrombotic state. It is possible that TGF-β also prevents expression of these structures. Regardless of the mechanism and range of the effect of TGF-β, our results demonstrate that the adhesive phenotype of human endothelial cells is capable of being inhibited. This phenomenon may be clinically relevant at sites of trauma, injury, or reperfusion

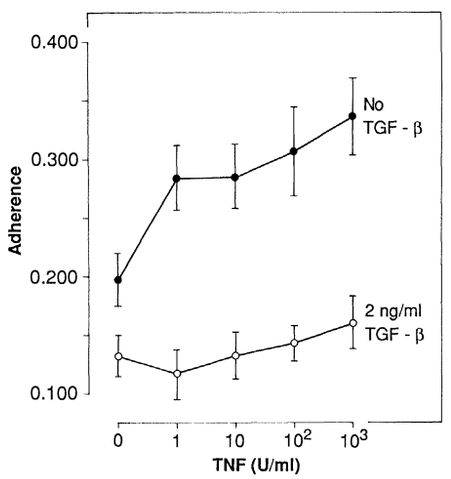

Fig. 2. TGF-β inhibits neutrophil attachment to unstimulated and TNF-α–stimulated endothelial cells. Endothelial cells cultured for 3 to 7 days were harvested by trypsinization and replated in 96-well microtiter trays with or without TGF-β (2 ng/ml). Various concentrations of recombinant human TNF-α (lot 3056-55, Genentech) were added for the final 4 hours of culture before being assayed for neutrophil adherence. The mean adherence ± SEM of 15 determinations from five separate experiments is given. All groups receiving TGF-β differed significantly from those not pretreated with TGF-β ($P \leq 0.03$). At all concentrations of TNF-α tested, adherence values for each group differed significantly from control value receiving no TNF-α ($P \leq 0.05$). However, all groups receiving TGF-β and TNF-α showed no significant increase in adherence when compared to groups receiving TGF-β alone.

Table 1. The effect of time of addition of TGF-β on polymorphonuclear cell (PMN) attachment to HUVEs. Endothelial cells harvested by trypsin from 4- to 6-day cultures and replated into microtiter wells at 1.5×10^4 cells per well were incubated with TGF-β (2 ng/ml) for various times and cultured for a total of 24 hours before assaying for neutrophil attachment. Time is the time (in hours) HUVEs were treated with TGF-β. Adherence is given as the OD_{570} of adhering neutrophils and HUVEs minus the OD_{570} of wells containing HUVEs alone. Inhibition is the percent inhibition of PMN binding. The *P* values for the difference between adherence in the presence of TGF-β and the control (no added TGF-β) are shown; NS, not significant. The mean (± SEM) of 7 to 12 determinations from three separate experiments is given.

Time (hour)	Adherence (OD_{570})	Inhibition (%)	*P* value
24	0.212 (± 0.010)	28	0.0002
18	0.237 (± 0.011)	19	0.003
6	0.253 (± 0.007)	14	0.007
3	0.259 (± 0.013)	12	NS
Control	0.294 (± 0.012)	—	

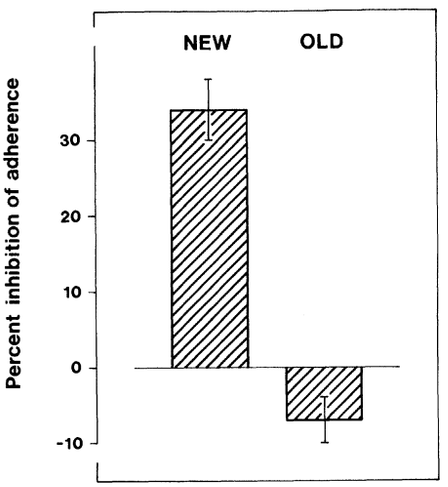

Fig. 3. Effect of age of endothelial cells on their response to TGF-β. After growth in culture for 3 to 6 days ("new" HUVEs) endothelial cells were harvested by trypsinization and replated at 1.5×10^4 cells per well into 96-well microtiter trays, with or without the addition of TGF-β (2 ng/ml) for 24 hours. The extent of neutrophil binding to the HUVEs was determined. A portion of these endothelial cells (0.2×10^6) were replated back into 25-cm^2 flasks for further growth in culture before being assayed at a later time. Three to 8 days (total time in culture 9 to 14 days, "old" HUVEs) later the cells were again harvested by trypsinization, replated into 96-well microtiter trays at 1.6×10^4 cells per well, and assayed for neutrophil attachment. The basal adherence to new HUVEs was 0.270 ± 0.030 and to old HUVEs was 0.256 ± 0.008 (arithmetic mean ± SEM, values not significantly different). The bars represent the mean percentage inhibition of adherence by TGF-β from four separate experiments, each performed in triplicate. The percentage inhibition of adherence after TGF-β treatment between new and old HUVEs differed significantly ($P = 0.0007$).

where vascular regeneration without excessive inflammation or thrombosis is essential.

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Ryanodine Receptor of Skeletal Muscle Is a Gap Junction–Type Channel

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In the sarcoplasmic reticulum membrane of skeletal muscle, the ryanodine receptor forms an aqueous pore identified as the calcium-release pathway that operates during excitation-contraction coupling. The purified ryanodine receptor channel has now been shown to have four properties usually associated with gap junction channels: (i) a large nonspecific voltage-dependent conductance consisting of several open states; (ii) an inhibition of open probability by low *p*H; (iii) an inhibition of open probability by calcium; and (iv) a sensitivity to blockade by heptanol and octanol but not other alcohols. This functional homology may provide an insight into the mechanism of how muscle cells transduce depolarization into an intracellular release of calcium.

COUPLING OF MUSCLE CELL EXCITATION to mechanical contraction occurs at the junction between the transverse tubular (TT) foldings of the plasma membrane and the membrane of the sarcoplasmic reticulum (SR), the intracellular organelle involved in cellular Ca^{2+} homeostasis. In skeletal muscle, the 450-kD ryanodine receptor has been identified as the major component of the "feet" structures that span the 100 Å cleft separating TT and SR (*1–3*). Using planar bilayer recording, we (*4, 5*) and others (*3, 6*) recently identified the purified 450-kD ryanodine receptor (Fig. 1A) as the Ca^{2+}-release channel of skeletal muscle. The purified channel displays a weak selectivity between Ca^{2+} and K^+ ions ($P_{Ca}/P_K = 6$; $P_{Cl} = 0$; where P is permeability) and a saturable conductance of 1 nS in K^+, 170 pS in Ca^{2+}, and about 100 pS in tris or choline (*5, 7*). Two characteristics of release channels, activation by ryanodine (*4–6*) and blockade by ruthenium red (*7*), are shown in Fig. 1C. As in native release channels (*4*), submicromolar ryanodine decreases the conductance and increases mean open time, driving the purified channel into state of quasi-permanent activation. Further addition of cis-internal 1 μM ruthenium red produces a flickery block and a marked decrease in the fraction of time spent open or the open probability (p_o). In this experiment (Fig. 1C) $p_o = 0.16$ in control, $p_o = 0.85$ after treatment with ryanodine, and $p_o = 0.18$ after addition of ruthenium red. Activation by adenine nucleotides (*7*), another characteristic of release channels, has been described elsewhere (*5*). Thus, the purified 450-kD receptor is a functional SR release channel.

Previous studies in SR vesicles have shown that molecules up to the size of glucose could permeate through the release channel (*8*). This broad permeability to electrolytes and nonelectrolytes and the fact that the open channels have several levels of conductance of large unit value (Fig. 1B) suggested a similarity to gap junctions. Channels formed by purified gap junction proteins have relatively large conductances that in physiological saline vary from 400 pS (2 nS in 1*M* KCl) in the bovine lens MIP-26 protein (*9*) to 140 and 280 pS in the rat liver 27 kD protein (*10*). Similar values have been reported in situ, in heart and lacrimal cells (*11*). In the case of the ryanodine receptor there appears to be at least three open states (Fig. 1B) with approximate conductances in 0.25*M* KCl of 800 pS, 400 pS (260 pS in 0.15*M* KCl), and 200 pS. The largest conductance (800 pS) is associated with the briefest lifetime (60 μs), whereas the lowest conductance (200 pS) is associated with the longest lifetime (8 ms). The 400-pS channel is most frequently observed and is thus the main contributor to the p_o. Thus, like junctional channel openings recorded with purified proteins (*9, 10*), the ryanodine receptor has one or more conductance states that pass large nonspecific currents.

The strongest homology to junctional channels was found in the effects of protons, Ca^{2+}, and 1-octanol, the well-known uncouplers of gap junctions (*12*). Over a narrow range, low *p*H closes the ryanodine receptor channel (Fig. 2A). We focused exclusively on the behavior of the most common 400-pS channel, which was present alone without other conductances in 78% of all recordings ($n = 48$). At *p*H 7.4 and *p*Ca 5, the average p_o is between 0.25 and 0.10 (average $p_o = 0.2$, SD = 0.05, $n = 10$). In this particular recording, $p_o = 0.14$. Reducing *p*H on either side of the channel results in a decrease in the frequency of openings and a shortening of lifetimes. Both parameters contribute to a decrease in p_o (Fig. 2D). Open probability drops from its near maximum value at *p*H 7.4 (normalized to 1) to almost null at *p*H 6.5. The absolute p_o at *p*H 6.5 was in the range $0.05 > p_o > 0.001$ (average $p_o = 0.03$, SD = 0.02, $n = 8$). The effect of *p*H was fully reversible within the range tested, and p_o reaches a maximum ($p_o = 0.35$) at *p*H values >7.6. The dose-response curve for *p*H titrations from either side (Fig. 2D) could be fitted with a cis *p*K value for H^+ of 7.2 (SD = 0.12, $n = 4$), a trans value of 7.1 (SD = 0.05, $n = 4$), and Hill coefficients for H^+ of (cis) 4 (SD = 1.04, $n = 4$) and

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