primers, since gel electrophoresis of the PCR products reveals a discrete band at 240 bp absent from the HLA deletion mutant (*7*) (Fig. 2). In addition, hybridization screening of the M13 clones from this amplification indicates that about 20% are homologous to the DQα probe, an increase of 20 times over the β-globin amplification. The basis for the difference in the specificity of amplification, defined as the ratio of target to nontarget clones, is not clear. It is likely to reflect the primer sequences and their genomic distribution rather than the different reaction conditions used (Fig. 2). The differences between the sequence of the β-globin primers GH18, GH19 and PC03, PC04 (Fig. 1) may account for the observed change in the gel profile of the PCR-amplified products (Fig. 2, lanes 1 and 2 versus lanes 3 and 4), reflecting some difference in the amplification of nontarget segments.

Three HLA DQα PCR clones derived from the homozygous typing cell LG2 were subjected to sequence analysis (*8*). Two clones were identical to a DQα complementary (cDNA) clone from the same cell line. One differed by a single nucleotide, indicating an error rate of approximately 1/600, assuming the substitution occurred during the 27 cycles of amplification. This procedure has also been used to analyze sequences from polymorphic regions of the HLA DQβ and DRβ loci.

Our rapid method for the cloning of specifically amplified genomic fragments relies on the ability of oligonucleotides to function as PCR primers with unpaired bases near their 5′ ends. In the later cycles of amplification these primers anneal primarily to the amplified products rather than to the original genomic sequences, and are therefore fully complementary. The β-globin linker-primers GH18 and GH19 appear to be approximately as efficient and specific as the fully matched primers. The HLA DQα linker-primers GH26 and GH27, with even more 5′ mismatches, show an amplification specificity 20 times higher than the β-globin primers. A large number of DQα clones were obtained from just 100 ng of this amplified genomic DNA. Our data suggest that the error rate over many cycles of amplification is sufficiently low so that reliable genomic sequences can be determined directly from PCR amplification and cloning.

This procedure offers significant advantages over standard cloning protocols for the analysis of sequence polymorphisms in that it circumvents the construction and screening of full genomic libraries and could potentially be initiated from nanogram amounts of DNA. It is capable of isolating only a limited region of the genome, however, and requires sequence information to identify conserved primer and probe segments. Unlike direct genomic sequencing (*9*), the cloning of amplified DNA allows the separation of related genes and alleles prior to sequencing and does not require a detailed knowledge of adjacent restriction sites. The application of oligonucleotide linker-primers to introduce specific restriction sites into PCR-amplified DNA may also prove useful as a general cloning strategy.

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IPTG, isopropyl-β-D-thiogalacto-pyranoside; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

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12. We thank C. Long for the sequencing of PCR amplified DNA's; C. Levenson, L. Goda, and D. Spasic for the synthesis of oligonucleotides; J. Sninsky, R. Saiki, and K. Mullis for critical review; and K. Levenson for the preparation of this manuscript.

21 April 1986; accepted 17 July 1986

Inhibition of Endothelial Regeneration by Type-Beta Transforming Growth Factor from Platelets

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Damage to the vessel wall is a signal for endothelial migration and replication and for platelet release at the site of injury. Addition of transforming growth factor-beta (TGF-β) purified from platelets to growing aortic endothelial cells inhibited [^{3}H]thymidine incorporation in a concentration-dependent manner. A transient inhibition of DNA synthesis was also observed in response to wounding; cell migration and replication are inhibited during the first 24 hours after wounding. By 48 hours after wounding both TGF-β–treated and –untreated cultures showed similar responses. Flow microfluorimetric analysis of cell cycle distribution indicated that after 24 hours of exposure to TGF-β the cells were blocked from entering S phase, and the fraction of cells in G_1 was increased. The inhibition of the initiation of regeneration by TGF-β could allow time for recruitment of smooth muscle cells into the site of injury by other platelet components.

THE CONTINUITY OF THE VASCULAR endothelium is lost as atherosclerotic lesions progress (*1*). In contrast, even in large wounds the endothelium rapidly regenerates both in vitro and in vivo (*2*). This raises the possibility that some property of the atheromatous wall or element of the blood interacting with the wall at the developing plaque can act to prevent the normal processes of endothelial regeneration. An obvious source of such an effect at sites of denudation is the platelet. Among the components released from platelets at a site of vascular injury are a group of growth regulatory proteins, including platelet-derived growth factor (PDGF), a growth factor similar to epidermal growth factor (EGF), and transforming growth factor-beta (TGF-β) (*3, 4*).

The endothelium grows as a strictly density-inhibited monolayer. Although endothelial cell growth requires serum or plasma, PDGF and EGF are not required (*5*). In the presence of acid-treated serum, aortic endothelial cells have been shown to form colonies in soft agar (*6*). Similar results were obtained from mouse embryo cells (AKR-2B) or normal rat kidney (NRK) cells and the activity that promotes growth in soft agar was identified as TGF-β (*4*). We report here that TGF-β purified from human platelets can inhibit the endothelial regeneration process by inhibiting both replication and migration.

Transforming growth factors were originally found in viral-transformed rodent cells, and it was postulated that they were involved in neoplastic cell growth (*7, 8*). However, their presence in normal tissues, including kidney, placenta, and platelets, suggests a more general function (*4, 9*). Platelets contain 40 to 100 times as much TGF-β as other nonneoplastic tissues. TGF-

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β has a different primary structure than TGF-α and consists of two identical peptide chains of 12,500 daltons cross-linked by disulfide bonds (*10*). TGF-β, in the presence of EGF, is a potent stimulator of growth in soft agar of AKR-2B or NRK cells (*11*). Under conditions of anchorage-dependent growth, TGF-β is not mitogenic for either cell line when assayed for 24 hours. However, DNA synthesis is stimulated in AKR-2B cells at 36 hours (*12*). EGF and insulin-stimulated DNA synthesis in AKR-2B cells is markedly inhibited at 24 hours by addition of TGF-β, but a delayed response is observed at 36 hours. In addition, Tucker and co-workers (*13*) have shown that a polypeptide growth inhibitor purified from conditioned medium from BSC-1 cells is essentially identical to TGF-β in molecular weight and biological activity. Certain lung and mammary tumor cell lines also show increased cell cycle times in monolayer culture and decreased anchorage-independent growth in the presence of TGF-β (*14*). These results indicate that TGF-β acts as a bifunctional regulator of cell growth.

Addition of aliquots derived from gel filtration and high-pressure liquid chromatography of an acid ethanol extract of human platelets (*4*) to subconfluent growing endothelial cells showed a single peak of activity inhibiting DNA synthesis, measured as the rate of incorporation of [^{3}H]thymidine (*15*). The inhibition was confirmed by whole-cell autoradiography, in which the percentage of labeled nuclei after treatment plateaued at 30% of the control value (*16*). This peak was coincident with that of TGF-β activity stimulating NRK fibroblasts to form colonies in soft agar in the presence of EGF (*7*). Experiments were carried out to compare the stimulation of colony formation of NRK cells in soft agar with the inhibition of DNA synthesis in subconfluent endothelial cells under conditions of anchorage-dependent growth (Fig. 1). The half-maximum stimulation of colony growth of NRK cells occurred at a concentration of TGF-β of 0.15 ng/ml and elicited a near maximal effect at 0.8 ng/ml. These results are similar to those obtained with TGF-β purified from human platelets by other groups (*4, 13*). In comparison, the half-maximum inhibition of endothelial cell growth was at 1.5 ng/ml. The inhibition of BSC-1 cells by TGF-β appears to be more sensitive than that of endothelial cells with a half-maximum at 0.15 ng/ml (*13*). The reason for this difference is not known. Perhaps it reflects a difference between established cell lines and endothelial cells, which retain a limited replicative life span.

To test the effect of TGF-β on wound edge regeneration, we removed a portion of a confluent culture of aortic endothelial cells by mechanical abrasion (Fig. 2). The cells were allowed to regenerate in the presence of TGF-β; [^{3}H]thymidine was present continuously. Autoradiographic analysis shows that approximately 68% of the control cells migrating over the wound line had entered DNA synthesis in the first 24 hours after addition of TGF-β (Table 1). Previous studies (*17*) have shown that endothelial cells at the leading edge of a wound begin DNA synthesis at about 8 to 10 hours and reach a maximum at 20 hours. As expected at 24 hours, the labeling index of the cells in the leading edge was inhibited nearly 37% by addition of TGF-β (6.4 ng/ml). The distance migrated by the cells was also reduced to 41% (Table 1). However, the labeling index of the cells of the leading edge at 48 hours was approximately the same in the TGF-β–treated culture as in the control culture. Similar results were obtained when

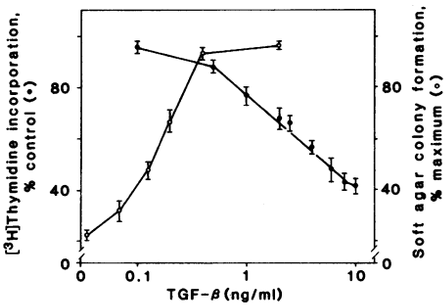

Fig. 1. Comparison of inhibition of [^{3}H]thymidine incorporation and colony formation in soft agar by TGF-β. Purification of TGF-β from outdated human platelets was as described previously (*4*). After gel filtration of acid-ethanol–solubilized platelet proteins, TGF-β was purified by two successive high-pressure liquid chromatography steps with the use of μBondapak C_{18} and CN columns (Waters Associates). The columns were eluted with acetonitrile and *n*-propanol gradients, respectively. The mobile phase for both columns was 0.05% trifluoroacetic acid. A single peak of anchorage-independent growth-stimulating activity eluting at 35% *n*-propanol, considerably after the elution of contaminating platelet peptides, was seen from the final step. Aliquots were removed from the column fractions, 10 μg of sterile carrier bovine serum albumin (BSA; Pentex) was added, and the samples were lyophilized to dryness. Samples of the peak fraction were dissolved in Dulbecco's phosphate-buffered saline (PBS) and assayed for their ability to stimulate colony formation of NRK fibroblast cells in 0.3% agar in the presence of EGF (1.5 ng/ml) (*8*) (○) or [^{3}H]thymidine incorporation in exponentially growing bovine aortic endothelial cells (●). Endothelial cells (1×10^4) were plated in 2-cm^2 wells in 0.5 ml of Waymouth's medium containing 10% adult bovine serum (Hyclone). After 48 hours of incubation, samples of the peak fraction were added in triplicate; 24 hours later the cells were labeled for 2 hours by addition of 1 μCi/ml [^{3}H]thymidine (*15*). Points are averages of triplicates and error bars are SEM's.

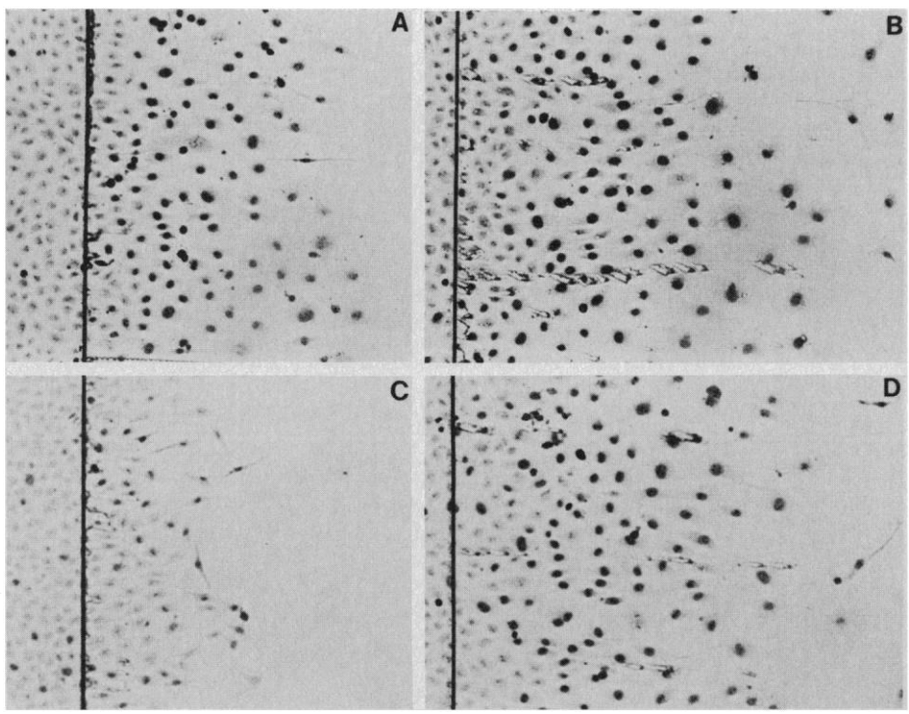

Fig. 2. Autoradiographs of a wound in confluent endothelial cell cultures. TGF-β was added at the time of wounding. The wound site is indicated by the vertical line. The cultures were exposed continuously to [^{3}H]thymidine (0.1 μCi/ml). (A) Control at 24 hours; (B) control at 48 hours; (C) TGF-β (6.4 ng/ml) added for 24 hours; (D) TGF-β (6.4 ng/ml) added for 48 hours.

the medium was changed at 24 hours and TGF-β was added again. At 48 hours, the migration distance of the cultures with TGF-β added was nearly the same as that of the control. The labeling index of cells in the intact sheet distant from the wound edge was approximately 3% at 24 hours and was not affected by addition of TGF-β. No change in morphology of the regenerating endothelial cells or the cell sheet was observed.

Analysis of the effect of TGF-β on cell cycle as measured by flow microfluorimetry is shown in Fig. 3. Determination of cell cycle compartments (*18*) from a confluent culture of aortic endothelial cells had 4% of its cells in S phase and 12% in G_2. In an exponentially growing culture, 25% of the cells are in G_2 and 32% are in S. Treatment of a growing culture with TGF-β for 24 hours reduced S to 9% while the percentage of cells in G_2 was not significantly changed at 28%. Exposure of endothelial cells to TGF-β for 48 hours increased the percentage of cells in S compared to that after 24 hours of exposure, and the percentage of cells in G_2 was unchanged compared to the control value at 48 hours. Apparently, TGF-β delays the entry of regenerating endothelial cells into S but, once the cells have passed the G_1/S border or are in G_2, they are no longer responsive to TGF-β.

The possible role of a transient inhibitory effect of TGF-β on endothelial regeneration in atherogenesis and wound healing is intriguing. Normally small areas of endothelial denudation are healed too rapidly to allow any stimulation of smooth muscle cell proliferation (*2*). Despite this, the advancing atherosclerotic lesion in hyperlipidemic animals does show evolution of small denuded areas (*1*). At a site of endothelial denudation, components released from platelets interact with the vessel wall within a few minutes (*19*). Persistent injuries may prolong the period of time platelets interact with the surface, increasing access of material released from platelet α-granules. Since TGF-β stimulates wound healing (*20*), the observed inhibition may be important for recruitment of smooth muscle cells into the wound by the platelet-released proteins, such as PDGF or a heparin endoglycosidase (*21*). When areas of the endothelium larger than 100 cells wide are removed, the endothelium fails to complete regeneration (*22*). This suggests that a certain delay or distance of endothelial regeneration is required for remodeling the vessel wall at a site of injury.

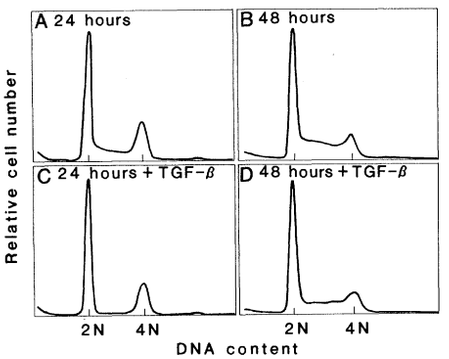

Fig. 3. Distribution of cell cycle phases among exponentially growing endothelial cells after exposure to TGF-β. The cells (10^5) were plated in 60-mm dishes in 3 ml of medium containing 10% adult bovine serum 24 hours before addition of TGF-β. After incubation for varying times, cell cycle analysis was performed by flow microfluorimetry. The cells were detached with 0.05% trypsin in PBS containing 0.54 m*M* EDTA. The cell pellet was suspended in diamidinophenylindole (10 μg/ml) in tris-buffered saline (*p*H 7.0) containing 0.06% Nonidet P-40, 1 m*M* $CaCl_2$, 21 m*M* $MgCl_2$, and 0.2% BSA. DNA content was analyzed on an ICP-22 flow cytometer (Ortho Diagnostic Systems), and representative distributions from four independent experiments are shown. Cell cycle compartments were estimated by an adaptation of previously described methods (*18*), using a nonlinear least-squares technique for curve fitting and subtraction of an exponential background noise. Fitted values for percentages of cells in G_1, S, and G_2 plus M, respectively, were (A) for 24 hours = 43, 32, and 25; (B) 48 hours = 46, 41, and 13; (C) 24 hours with TGF-β (12.5 ng/ml) = 63, 9, and 28; and (D) 48 hours with TGF-β = 54, 34, and 12. A typical confluent culture of aortic endothelial cells shows values of 84, 4, and 12.

Table 1. Effect of TGF-β on endothelial regeneration. Cells were first grown to stationary density in two-chamber Lab-Tek slides (Miles), and wounds were made with a 1-cm-wide fragment of a stainless steel razor blade drawn laterally across the dish surface. The cultures were rinsed once with PBS to remove nonadherent cells, and fresh medium (1.5 ml) containing TGF-β was added. The cultures were continuously labeled with [^{3}H]thymidine (0.1 μCi/ml), fixed, and prepared from autoradiography (*15*). Cell migration distance was measured from the razor mark at the initial line of injury to the nucleus of the cell farthest from the wound line at ten random locations along the wound line. Data are from autoradiographs of duplicate treated or untreated cultures and are shown as the means ± SEM.

Regeneration time (hour)	TGF-β (ng/ml)	Percent labeled nuclei		Migration distance (μm)
		Wound	Confluent layer	
24	0	68 ± 2	2.4 ± 0.2	8.4 ± 0.4
	3.2	56 ± 4	2.4 ± 0.4	6.9 ± 0.4
	6.4	43 ± 3	2.6 ± 0.4	5.0 ± 0.3
24 + 24*	0	60 ± 2	2.8 ± 0.3	15.3 ± 1.2
	3.2	62 ± 3	2.4 ± 0.3	15.1 ± 1.3
	6.4	59 ± 3	3.0 ± 0.4	15.2 ± 1.3
48	0	67 ± 2	5.3 ± 0.8	16.1 ± 1.2
	3.2	68 ± 3	6.1 ± 0.7	15.3 ± 1.4
	6.4	67 ± 5	7.1 ± 1.1	14.7 ± 1.1

*In this set of experiments, the medium was removed at 24 hours and replaced with fresh medium containing TGF-β and [^{3}H]thymidine.

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23. We thank the Puget Sound Blood Center for providing outdated platelets. We also thank J. Ranchalis and I. Werny for technical assistance. Supported by National Institutes of Health grant HL-18645.

2 January 1986; accepted 12 May 1986