Hypoxia Upregulates the Synthesis of TGF-β1 by Human Dermal Fibroblasts

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In this report, we have investigated the secretion and synthesis of transforming growth factor-β1 (TGF-β1) by human dermal fibroblast cultures in response to hypoxia (2% oxygen), and have compared it to standard oxygen culture conditions (15% oxygen at the cell surface). Sandwich enzyme-linked immunosorbent assay (SELISA) showed a selective and progressive increase in secretion of the TGF-β1 isoform in response to hypoxia, up to ninefold after cultures were exposed to low oxygen for 72 h; TGF-β2 peptide levels were not increased. We then investigated the transcriptional regulation of the TGF-β1 gene in response to low and standard oxygen tensions. In the first 24–48 h, TGF-β1 mRNA levels decreased steadily in both oxygen environments. This mRNA decline continued for up to 72 h in standard oxygen but not in cultures exposed to low oxygen tension. At 72 h, steady-state TGF-β1 mRNA levels were 8 times greater in low compared to standard oxygen, and this increase was reversible upon re-exposure of fibroblast cultures to standard oxygen tension for 24 h. Elevated TGF-β1 m-RNA levels in both low and standard oxygen declined steadily and with the same half-life after the addition of actinomycin D, suggesting that hypoxia increased TGF-β1 transcription rather than mRNA stability. We conclude that low oxygen tension upregulates the synthesis of TGF-β1 by human dermal fibroblasts, and leads to increased secretion of this peptide. *J Invest Dermatol 97:634–637, 1991*

The transforming growth factor (TGF-β) gene family includes several structurally homologous proteins with variable effects on cell growth and differentiation (for reviews, see [1–3]). TGF-β1, the first of these peptides to be discovered, is known to be an important mediator of tissue injury and repair. It is secreted by most cells, suggesting that regulation of its synthesis is an essential step. In this study, we have examined the synthesis of TGF-β1 by human dermal fibroblast cultures exposed to hypoxia, a condition known to occur during tissue repair [4] and in fibrotic skin [5]. We have hypothesized that a low-oxygen tension, as observed in these conditions, may cause fibroblasts to produce greater than normal amounts of TGF-β1. The results reported here support this hypothesis.

MATERIALS AND METHODS

Fibroblast Cultures Human dermal fibroblasts from the dorsal forearm skin of healthy donors were isolated as previously described [6]. Four different strains in passages 3–8 were used throughout the experiments. Cultures were grown in T-75 flasks (Corning Glass Works, Corning, NY) and seeded in DMEM (Sigma, St. Louis, MO) and 10% fetal bovine serum (FBS; GIBCO Laboratories, Grand Island, NY). Fibroblasts were grown and kept in 5% CO_2, 95% air at 37°C. These culture conditions were achieved in a Forma (Forma Scientific, Marietta, OH) incubator and are widely considered standard conditions for fibroblast growth. For experiments, fibroblast cultures were grown in standard oxygen conditions until near confluent. At this point, replicate sets of flasks were transferred to a separate incubator (Forma) set at 2% oxygen, a concentration achieved by infusion of nitrogen. Cell counts were done with a hemacytometer. The actual oxygen concentrations at the cell monolayer surface in the two different oxygen environments were measured with a needle probe (Lazar, Los Angeles, CA). Experiments were done by placing replicate cultures in either 2% oxygen or in standard oxygen for various periods of time up to 72 h; conditioned media were collected and RNA was isolated (see below) from two T-75 flasks for each time point. In other experiments, cultures were left for 48 h in either low or standard oxygen, at which time they were incubated with 5 μg/ml of actinomycin D (Sigma) for up to 6 h.

TGF-β1 and β2 Protein Measurements Fibroblasts were grown to near confluence in DMEM with 10% FBS. The medium was then changed to DMEM supplemented with 100 μg/ml of BSA fraction V (Sigma) with five changes of medium over 5 h to eliminate carryover of serum TGF-β. Fresh DMEM with 100 μg/ml BSA fraction V was added and allowed to condition for from 24 to 72 h, with a medium change every 24 h. A total of 5 ml of medium was used to condition cells in 75-cm^2 flasks. At the end of the appropriate incubation times, in either standard or low oxygen, conditioned media were collected and supplemented with 2 μg/ml aprotinin, leupeptin, pepstatin A, and 120 μg/ml of phenylmethylsulfonyl fluoride (PMSF). Samples were then stored at −70°C. Cell counts were done on the cultures.

Measurements of TGF-β1 and β2 in duplicate samples of conditioned media were done using enzyme-linked immunosorbent assays (SELISA) for TGF-β1 and β2 using both turkey and rabbit

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Abbreviations:
PDGF: platelet-derived growth factor
SELISA: sandwich enzyme-linked immunosorbent assay
TGF-β: transforming growth factor-β

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neutralizing polyclonal antibodies against native TGF-β. Each assay is based on the binding of two different antibodies to distinct epitopes of the peptides. These assays have been previously shown to measure TGF-β1 and β2 quantitatively and specifically in complex biologic fluids, with detection limits of 2–5 pg [11]. TGF-β3 and β5 either do not cross-react or cross-react very poorly in these assays. TGF-β1.2 heterodimer, although neutralized up to 80% by either TGF-β1 and β2 antibodies, shows only a 1.5 and 3.7% cross-reactivity in the TGF-β1 and β2 SELISA, respectively. Details concerning preparation and specificity of the antibodies as well as the methods for handling conditioned media for these SELISA have been previously published [7,8].

RNA Extraction and Northern Analysis Total RNA from cells was isolated by extraction in guanidinium isothiocyanate [9]. RNA was separated for Northern blot analysis on 1.2% agarose gels containing 2.2 M formaldehyde, and transferred to Nytran membrane (Schleicher & Schuell, Keene, NH) in 1.0 M NH_4Ac. A 218-bp ^{32}P-labeled single-stranded probe complementary to the mature protein coding region of the human TGF-β1 cDNA [10] was used for Northern blot analysis. The probe was prepared by primer extension on a single-stranded M13 template containing a 243-bp (Pvu II) insert from the 3′ end of the human cDNA. A specific oligonucleotide within the insert was extended using the Klenow fragment of DNA polymerase, and the resulting product was digested with Xba I at a unique site in the polylinker region to remove M13-specific sequences [11]. The single-stranded DNA probe was isolated from a 5% acrylamide gel run under denaturing condition. For RNA electrophoresis, 10–15 μg of total RNA were loaded per lane, as measured by absorbance at 260 and 280 nm. Ethidium bromide staining was used to assess the relative amount and the intact nature of the RNA. Northern hybridization was performed according to the method of Church and Gilbert [12] in a buffer containing 1% bovine serum albumin (BSA), 0.5 M sodium phosphate (pH 7.0), 7% sodium dodecyl sulfate (SDS), 0.01 M EDTA, and 2×10^8 cpm/ml TGF-β1 ssDNA probe at 65°C. The blots were washed 2 times in 40 mM sodium phosphate buffer (pH 7.0), 0.5% BSA, 5% SDS, and 0.01 M EDTA for 10 min at room temperature, 4 times in 40 mM sodium phosphate buffer (pH 7.0), 1% SDS, and 0.01 M EDTA for 10 min at 65°C. Autoradiograms of Northern Blot were quantitated with an LKB scanning densitometer.

RESULTS

Peptide Synthesis Cultures of human dermal fibroblasts were exposed to either standard or low oxygen tension as they approached confluence and were not actively dividing, with a typical cell count of 7×10^5 cells per 75 cm^2 flask. No difference was noted between the rate of cell growth and the passage number (3–8) in which cells were studied. Actual oxygen concentrations at the cell monolayer surface, as measured with a needle probe, were either 14 mm Hg (2% oxygen) or 94 mm Hg (15% oxygen) at sea level, and at 37°C. No obvious morphologic changes were observed by light microscopy in cells exposed to hypoxic conditions. As measured with a hemacytometer, cell counts did not differ significantly between cultures kept in standard conditions or made hypoxic. In a typical experiment in which cells were incubated for 72 h in 1% FBS, cell counts in two 75-cm^2 flasks were $7.2 \times 10^5 \pm 0.20$ (SD) in low oxygen and $7.1 \times 10^5 \pm 0.22$ in standard oxygen conditions. We have examined the effect of hypoxia on the secretion of TGF-β1 and β2 isoforms into conditioned media. Figure 1 indicates the amounts of TGF-β1 accumulating in conditioned media every 24 h over a period of 72 h; the media were removed and replenished every 24 h. It shows that TGF-β1 peptide synthesis was increased in hypoxic cultures, with up to a ninefold increase at 72 h over control (standard oxygen) cultures ($p < 0.05$); TGF-β2 levels were always less than 0.5 pM (data not shown) and did not increase. The results shown in Fig 1 were obtained in cultures in their fifth in vitro passage; similar results were obtained at the eighth passage.

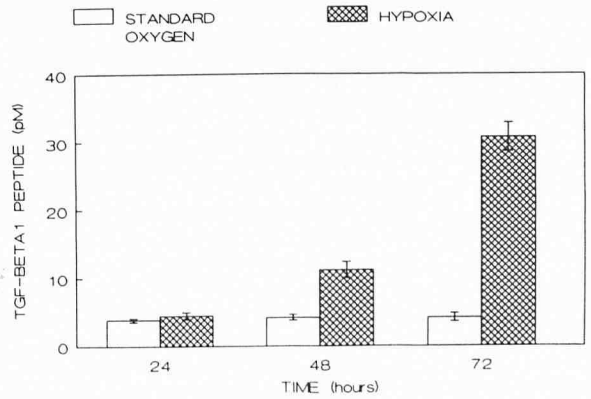


Figure 1. Levels of TGF-β1 peptide in conditioned media. Media were collected at specified times after cultures were either exposed to hypoxia (2% oxygen) or left in standard (15%) oxygen. Cultures of fibroblasts in 75-cm^2 flasks were first washed five times over 5 h with DMEM containing 100 μg/ml BSA, and were then conditioned with the same media (see *Materials and Methods*). Results represent the mean ± SD of duplicate flasks.

Transcriptional Regulation We then investigated the transcriptional regulation of the TGF-β1 gene in response to low and standard oxygen tensions. As shown by Northern blot analysis in Fig 2, where the single band represents the 2.4-kilobase pair TGF-β1 transcript, TGF-β1 steady-state mRNA levels declined steadily in both oxygen environments during the first 24 h. Figure 2 shows that this effect occurred in cultures treated with 10% or 1% FBS, though it was more obvious with 1% FBS. As seen in Fig 3*A*, this mRNA decline continued up to 72 h in standard oxygen conditions; a similar decrease was observed in five experiments in which mRNA was measured. In contrast, as shown in Fig 3*A*, in cultures exposed to low oxygen tension, an increase in TGF-β1 mRNA was observed after the first 48 h. Ethidium bromide staining confirmed that RNA loading was similar in all lanes (Fig 3*B*). Similar mRNA results were obtained when cultures were exposed to low or standard oxygen in the presence of 10% FBS (Fig 3*A*) or in the absence of serum (Fig 4). At 72 h (Fig 5), steady-state TGF-β1 mRNA levels in cultures treated with 1% FBS were eightfold greater in hypoxia than in standard oxygen (control), as measured by densitometric analysis. Upregulation of TGF-β1 mRNA levels in response to low oxygen

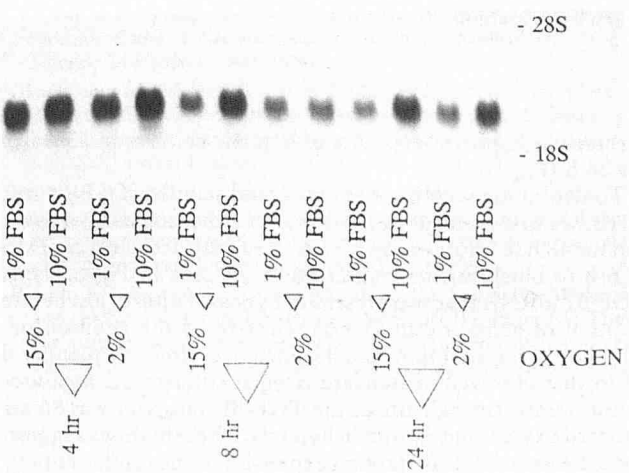
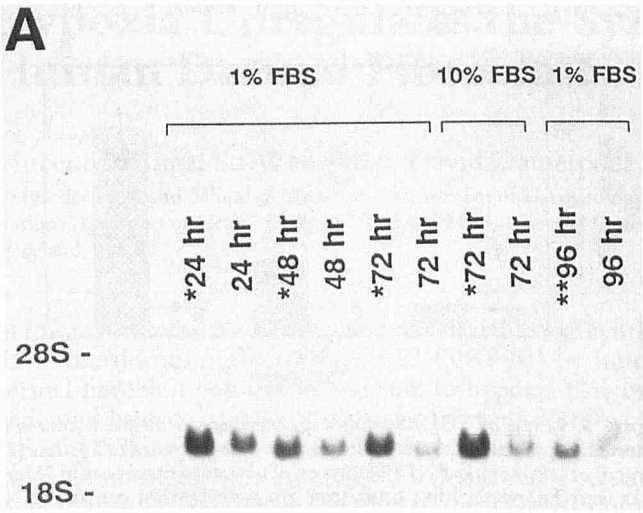


Figure 2. Northern blot of TGF-β1 mRNA in human dermal fibroblast cultures. Cultures were either exposed to hypoxia (2% oxygen) or left in standard (15%) oxygen, and these oxygen concentrations are those measured at the cell surface with a needle probe. TGF-β1 mRNA levels were measured in cultures supplemented with either 1% or 10% FBS for 4, 8, and 24 h.



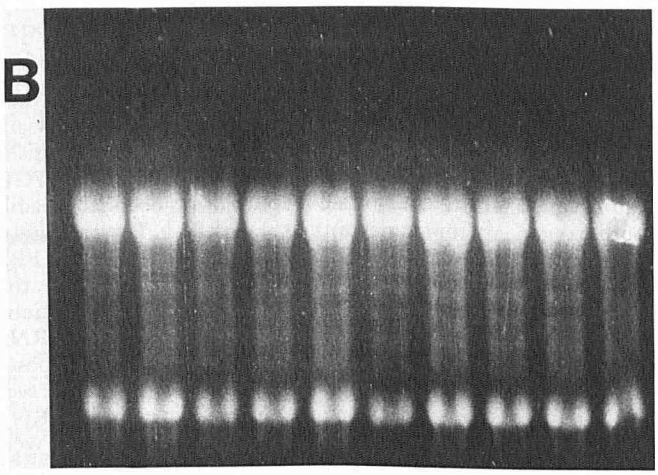


Figure 3. Northern blot of TGF-β1 mRNA in human dermal fibroblast cultures in hypoxia or standard oxygen. *A*, Time periods with an * indicate cultures exposed to hypoxia for the specified period of time. The *second lane from the right,* labeled with **, indicates cultures exposed to hypoxia (2% oxygen) for 72 h and returned to standard (15%) oxygen for an additional 24 h. The *last lane from the right* shows cultures kept concurrently in standard oxygen for a total of 96 h. *B*, Ethidium bromide-stained blot shown in *A* before hybridization.

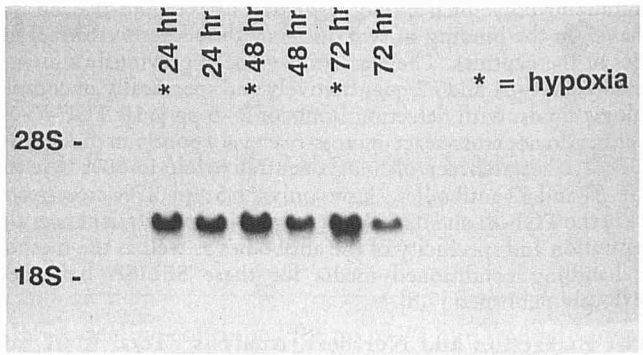


Figure 4. Northern blot of TGF-β1 mRNA in the absence of serum. Cultures of human dermal fibroblasts were exposed either to low (2%) or standard (15%) oxygen. *Cultures exposed to hypoxia.

was reversed upon re-exposure of hypoxic cultures to 15% oxygen for 24 h (Fig 3*A*, 5).

To determine whether hypoxia could stabilize TGF-β1 mRNA levels, we first preincubated cultures in either standard or low oxygen for 48 h. Cultures were then treated with actinomycin D for up to 6 h to block further transcription. As seen in Fig 6, increased TGF-β1 mRNA that was present in hypoxic cultures just before the addition of actinomycin D was sensitive to the addition of this inhibitor of transcription, and declined over time in a manner similar to that observed in standard oxygen cultures. As measured by densitometry, the half-life of the TGF-β1 transcript was 86 min in standard oxygen and 83 min in hypoxia. These findings suggest that the increase in TGF-β1 protein expression is the result of upregulation of TGF-β1 transcription by hypoxia.

DISCUSSION

Fibroblasts play a fundamental role in fibrosis and tissue repair, where they are responsible for the synthesis of collagen and other extracellular matrix macromolecules. During the first few days after wounding, fibroblasts that have migrated to the site of injury function in an environment virtually devoid of oxygen [44]. It is likely that the secretory response of wound fibroblasts is strongly influenced by low oxygen tension [13]. In this report, we have studied the regulation of TGF-β1 expression and secretion by human dermal fibroblast cultures exposed to hypoxic conditions. We have found that prolonged exposure to hypoxia upregulates TGF-β1 mRNA levels and causes a dramatic and selective increase in the secretion of the TGF-β1 isoform. As shown by our results with actinomycin D, these findings can best be explained by an increased transcriptional activity of the TGF-β1 gene by hypoxic conditions.

An interesting and previously unreported finding was the progressive decrease in TGF-β1 mRNA levels, which was evident at 8 h and continued at sequential time points over a period of 72 h in cultures left in standard oxygen conditions; hypoxic cultures showed a similar but slower decline and only in the first 48 h. These decreases in mRNA occurred both in high and low serum conditions as well as in the absence of serum, suggesting that they may not be due to availability of essential serum components. Although the addition of TGF-β1 to cells has been shown to increase TGF-β1 mRNA levels [11], most of the TGF-β1 peptide in serum is biologically inactive. As shown, exposure of fibroblast cultures to low oxygen resulted in up to a ninefold increase in TGF-β1 protein secretion into conditioned media. TGF-β2 synthesis was not increased, suggesting differential regulation of these two peptides by hypoxia. It should be noted that our experiments have addressed TGF-β1 synthesis and have not measured alterations in the amounts of biologically active

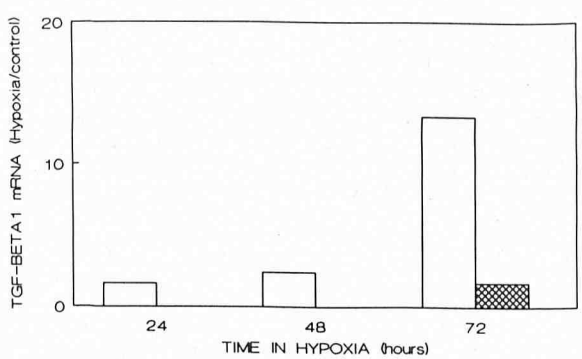


Figure 5. Densitometric analysis of TGF-β1 Northern blot shown in Fig 2 for cultures supplemented with 1% FBS. Measurements at the specified time periods are in arbitrary units and represent the ratio of mRNA levels in hypoxia to those in standard oxygen (control). *Hatched bar,* measurements made after cultures were exposed to low oxygen for 72 h and returned to standard oxygen for an additional 24 h.

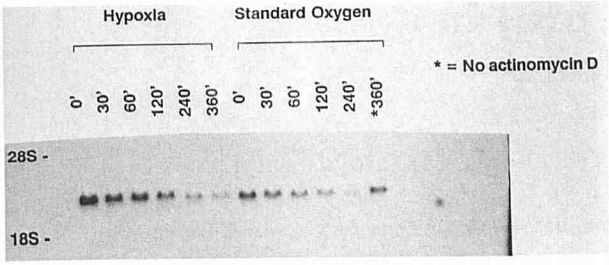


Figure 6. Northern blot of TGF-β1 mRNA in response to actinomycin D. Human dermal fibroblasts cultures were first preincubated in either hypoxia (2% oxygen) or standard (15%) oxygen for 48 h, and were then treated with actinomycin D (5 μg/ml). TGF-β1 mRNA was measured at the specified times after the addition of actinomycin D and cultures were kept in their respective oxygen environments. The *first lane from the right* is from cultures that did not receive actinomycin D.

peptide in response to low oxygen tension. TGF-β are secreted as larger precursor molecules that require cleavage to become biologically active [1,2].

The observations reported here with respect to upregulation of TGF-β1 mRNA and peptide synthesis in response to hypoxia may be due to increased TGF-β1 transcription or decreased TGF-β1 mRNA breakdown. Although we did not measure transcriptional rates directly, the fact that actinomycin D blocked the increase in mRNA levels strongly suggests that it is unlikely that low oxygen tension increased the half-life of TGF-β1 mRNA. However, the specific mechanisms responsible for increased transcription in hypoxia remain unknown. We do not know whether our findings are directly related to low oxygen, or whether changes in pH or cellular metabolism in response to low oxygen tension play a role.

Relatively little information is available on the effect of hypoxia on the expression of TGF-β1 or other genes. Kourembanas et al [14], studying hypoxic endothelial cells, reported increased transcription of a PDGF B chain mRNA and no change in TGF-β1 mRNA expression in cultures supplemented with 20% serum; the authors did not study endothelial cells in lower serum conditions and did not measure PDGF or TGF-β1 protein synthesis. Interestingly, hypoxic fibroblasts have been found to produce excessive mRNA levels of certain genes, including retrotransposon-related VL30 element RNA, deemed important in neoplastic processes [13].

The relevance of the in vitro findings reported here to in vivo hypoxia is at this time conjectural, but deserves brief comment. Low oxygen conditions have been reported in wounds [4] and in fibrotic skin [5], as well as with excessive aortic medial proliferation [15]. Indeed, TGF-β1 has been found in wounds [16] and in fibrotic skin [17,18]. Because TGF-β1 is a potent stimulus for collagen synthesis [19] and other extracellular matrix proteins [2], it is reasonable to hypothesize that an increased production of TGF-β1 by fibroblasts in response to hypoxia could be responsible in part for physiologic or pathologic increases in matrix accumulation.

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