York, 1987), chap. 4.7.
17. E. Winter, F. Yamamoto, C. Almoquera, M. Perucho, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7575 (1985).
18. We thank B. Peltre, M. Mutschler, and V. Schiff for technical assistance. Supported by grants from the CNRS, the INSERM (CNAMTS), the Ministère de la Recherche et de l'Enseignement Supérieur, the Fondation pour la Recherche Médicale Française, the Association pour la Recherche sur le Cancer, and the Ligue Nationale Française Contre le Cancer.

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Wound Macrophages Express TGF-α and Other Growth Factors in Vivo: Analysis by mRNA Phenotyping

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The presence of macrophages is required for the regeneration of many cell types during wound healing. Macrophages have been reported to express a wide range of mitogenic factors and cytokines, but none of these factors has been shown in vivo to sustain all the wound-healing processes. It has been suggested that transforming growth factor–α (TGF-α) may mediate angiogenesis, epidermal regrowth, and formation of granulation tissue in vivo. Macrophages isolated from a wound site, and not exposed to cell culture conditions, expressed messenger RNA transcripts for TGF-α, TGF-β, platelet-derived growth factor A-chain, and insulin-like growth factor–1. The expression of these transcripts was determined by a novel method for RNA analysis in which low numbers of mouse macrophages were isolated from wound cylinders, their RNA was purified and reverse-transcribed, and the complementary DNA was amplified in a polymerase chain reaction primed with growth factor sequence–specific primers. This single-cell RNA phenotyping procedure is rapid and has the potential for quantification, and mRNA transcripts from a single cell or a few cells can be unambiguously demonstrated, with the simultaneous analysis of several mRNA species. Macrophages from wounds expressed TGF-α antigen, and wound fluids contained TGF-α. Elicited macrophages in culture also expressed TGF-α transcripts and polypeptide in a time-dependent manner after stimulation with modified low-density lipoproteins and lipopolysaccharide endotoxin, which are characteristic of the activators found in injured tissues.

WHEN TISSUES ARE DAMAGED, A complicated process of healing takes place, the result of which is the replacement of dead tissue and fibrin with a scar. Macrophages are central to the wound-healing response, which requires the proliferation and migration of several regenerating cell types (*1*). In addition to debridement by macrophages, there is an ingrowth of blood vessels from the surrounding connective tissue, the proliferation of fibroblasts (which produce collagen), and the rapid proliferation and migration of epithelial cells over the broken surface. Ablation of macrophages slows the wound-healing response (*2*). Previously characterized mitogenic factors derived from macrophages in culture include interleukin-1 (IL-1), platelet-derived growth factor (PDGF), basic fibroblast growth factor (FGF), colony-stimulating factors (for monocytes, M-CSF; for granulocytes, G-CSF; and for granulocytes and monocytes, GM-CSF), bombesin, and transforming growth factor–β (TGF-β) (*3*, *4*). Tumor necrosis factor–α, an inflammatory mediator produced by macrophages, was recently reported to be angiogenic (*5*), but others have concluded that this effect is indirect (*6*). Transforming growth factor–α (TGF-α) and epidermal growth factor (EGF) bind to the EGF receptor with the same affinity (7). It has recently been reported that TGF-α or EGF acts through a direct, noninflammatory mode to direct the wound-healing processes in vivo (*8*). Wound fluid, conditioned by macrophages and other cells in vivo, is a rich source of mitogenic and angiogenic activity (*9*). It is not, however, clear which growth factors are actually expressed in vivo, and whether the macrophages recruited to these sites are the source of these polypeptides. We undertook a study to determine whether wound macrophages express growth factors in vivo.

To test our hypothesis, we developed a method for assaying the transcriptional phenotype of small numbers of cells or small amounts of mRNA. This method is superior in ease, speed, sensitivity, and resolution to RNA analysis by in situ hybridization, RNA blot analysis, and the nuclease protection assay for the study of short-lived, low copy number mRNA transcripts. It can be performed in 1 to 2 days from cell to analysis. The method consists of a microprocedure for isolating RNA from one to a few thousand cells, followed by two coupled enzymatic steps (*10*, *11*). The whole cellular RNA is first reverse-transcribed, and then cDNA subfragments are amplified by specifically primed polymerase chain reactions. Each specific cDNA subfragment can be visualized on agarose gels by ethidium bromide staining. Since several mRNA species can be assayed simultaneously, we call the method "single-cell mRNA phenotyping."

Glass-adherent cells ($\sim 1 \times 10^2$ to 2×10^2 per cylinder, 50 to 80% macrophages) were isolated from subepidermal wound cylinders (*9*) 6 days after implantation in mice; the RNA was purified and reverse-transcribed, the cDNA was divided, and cDNA subfragments were amplified by sequence-specific primers (Table 1). Products of the combined reverse transcription–polymerase chain reaction (RT-PCR) were fractionated by electrophoresis in agarose, stained with ethidium bromide, and validated by matching predicted size (Fig. 1A) by means of restriction enzyme analysis (Fig. 1B) or DNA blot analysis (*12*). The method resolves threefold differences in input RNA over a range greater than two orders of magnitude (Fig. 1C). Therefore, an exogenous polyadenylated cRNA (copy RNA synthesized as a sense strand from a pBR322/IL-1 construct plasmid by T7 polymerase) dilution series can be used as an internal standard to quantify endogenous mRNA transcripts of low copy number (Fig. 1C). With this method we can detect β-actin in a single peritoneal macrophage (Fig. 1D) and in less than 100 copies of cRNA transcripts (*11*).

We found that the adherent cells isolated from wound cylinders contained transcripts for TGF-α, TGF-β, PDGF-A, EGF, and insulin-like growth factor–1 (IGF-1) (Fig. 2A). In three of six wound cylinder preparations assayed, IL-1α was weakly expressed, perhaps owing to variable lipopolysaccharide endotoxin (LPS) contamination of the cylinders. Interleukin-1α was readily demonstrable in cultured macrophages stimulated with LPS (Fig. 1A). Transcripts of IGF-1 were also found in the P388D1 macrophage line (*12*). We did not, however, find EGF transcripts in macrophage cell lines or in highly purified cultured macrophages (*12*), which suggests that EGF was the product of a contaminating cell type. In

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parallel experiments, we analyzed adherent wound cylinder cells for growth factor antigens. Half of the cells had macrophage morphology, and most of the rest were polymorphonuclear leukocytes. The macrophages had TGF-α immunofluorescence signals in the absence, but not the presence, of soluble competing TGF-α (Fig. 2B). Similar adherent cells were positive for the macrophage-specific antigen F4/80 (Fig. 2B). About half of the wound cylinder cells with macrophage features were also positive for PDGF antigen by immunofluorescence (*12*). Wound fluid from the cylinders contained about 51 ng of TGF-α antigen per liter, as assayed by the enzyme-linked immunoabsorbent assay (ELISA) (*13*). Rabbit wound fluid contained 75 ng of TGF-α/EGF per liter, as measured by a

Table 1. Oligonucleotides used in RNA phenotyping amplification. Sequences are from published sources (*17*).

Transcript	Location of oligonucleotides in nucleotide sequence	
	5′ Oligonucleotide	3′ Oligonucleotide
β-Actin	25–44	269–245
TGF-α	58–78	297–277
TGF-β	1277–1296	1521–1502
PDGF-A	622–645	848–826
EGF	3953–3972	4200–4181
NGF-β	248–267	649–630
IL-1α	303–324	680–661
IGF-1	217–239	440–416

Table 2. Growth factors produced by LPS-stimulated P388D1 macrophages. P388D1 macrophages (1×10^8) were treated with 10 μg of LPS per milliliter for 48 hours in serum-free medium. The conditioned medium (100 ml) was dialyzed and concentrated as described in Fig. 4B. Ten milliliters of 10× concentrate in PBS was applied to a heparin-Sepharose column (Pharmacia) and eluted with a continuous gradient of 0 to 3.3*M* NaCl in PBS. Column fractions were dialyzed against PBS and assayed for mitogenic stimulation of Balb/c 3T3 cells (*19*). Mitogenic fractions eluting with the NaCl gradient were pooled and assayed for TGF-α/EGF by radioreceptor assay (*14*) and for PDGF, TGF-β, and basic FGF by dot immunoassay (*23*). The data are expressed as amount per 100 ml of conditioned medium. TGF-β was measured in several different column runs, and the range of activities in relative units is shown. The pool containing TGF-β (1.1 to 1.4*M* NaCl) had negative mitogenic activity (below the negative control). ND, not determined.

Eluting NaCl (*M*)	Mitogenic activity (% of total)	TGF-α/EGF (ng/100 ml)	FGF (ng/100 ml)	PDGF (ng/100 ml)	TGF-β (relative units)
0.2	33	31	0	0	0–5
0.4–0.7	23	0	0.1	25	0
1.1–1.4	−7	ND	ND	ND	95–100
1.7–2.1	12	0	1.0	0	0
2.2–2.4	9	0	5.0	0	0

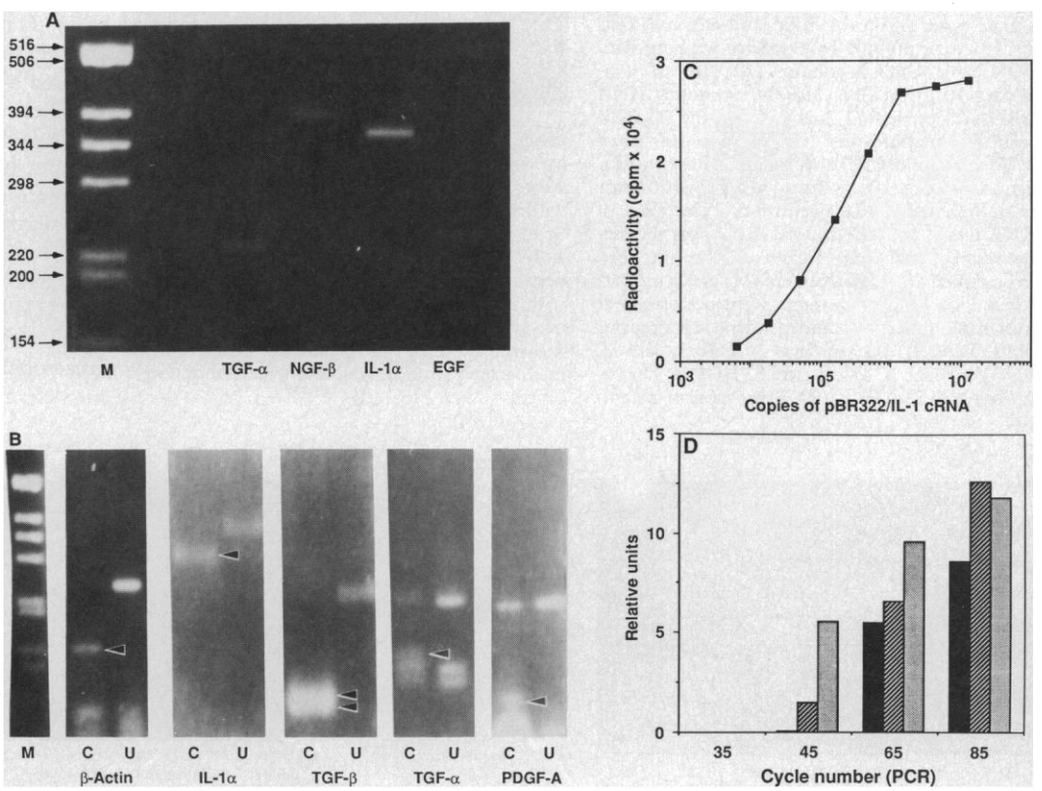

Fig. 1. (**A**) Fragments of predicted size. Fragments generated from cDNA were generated from known sources of mRNAs (*3, 4, 17*) by each sequence-specific primer pair. RNA was isolated from: mouse brain for TGF-α; thioglycollate-elicited peritoneal macrophages (TEPM) stimulated with LPS for 3 hours for IL-1α; Balb/c3T3 fibroblasts for NGF-β; and male mouse submaxillary glands for EGF (*10*). Whole RNA (0.1 μg) was reverse-transcribed and then amplified for 60 cycles of PCR (*10*). Arrows indicate sizes of a Hae III digest of φX174 in the marker lane (M). Each band is the size predicted for the amplified cDNA. Lack of a second higher band indicates that the RNA was not contaminated with genomic DNA. (**B**) Restriction enzyme analysis of DNA fragments generated from RT-PCR. Forty microliters of ethanol-precipitated RT-PCR fragments were digested with the restriction enzymes according to the manufacturers' instructions (Bethesda Research Laboratories and Promega Biotech). Paired digested (C) and undigested (U) fragments were fractionated by electrophoresis on a 4% agarose gel and stained with ethidium bromide. The enzymes used and diagnostic fragments are β-actin (Acc I), 151 bp; IL-1α (Pst I), 267 bp; TGF-β (Sma I), 125 + 119 bp; TGF-α(Sph I), 159 bp; PDGF-A (Acc I), 129 bp. Size markers (M) are as indicated in Fig. 1A. (**C**) Resolution of threefold differences in input cRNA by RT-PCR. The cRNA was produced by T7 polymerase from a pBR322/IL-1 construct plasmid (*24*) as template, purified by oligo(dT) chromatography, and quantified by absorbance at 260 nm. Serial threefold dilutions of cRNA were added to 1 μg of carrier RNA, so that the RT mixture contained 3×10^4 to 1.3×10^7 copies of cRNA. After RT, the cDNA was amplified by 40 cycles of PCR in the presence of [^{32}P]dCTP to follow incorporation into the specific cDNA fragment band. After separation on agarose gels, bands were excised and radioactivity was determined. (**D**) Generation of a β-actin signal by RT-PCR from a single macrophage. RNA, isolated as described in Fig. 2A, from 1 (filled bar), 10^2 (hatched bar), and 10^4 (dotted bar) TEPM was reverse-transcribed, and the cDNA was amplified by PCR. PCR products sampled at 35 to 85 cycles were separated by electrophoresis in 4% agarose gels, stained with ethidium bromide, photographed, and scanned with a Bio-Rad densitometer. Peak areas are given as relative units. An actin signal was routinely seen ($n > 20$) in <10 pg of total cellular RNA.

competitive radioreceptor assay (*12, 14*). These data provide evidence that adherent cells from wounds express TGF-α mRNA and that F4/80-positive wound macrophages express TGF-α antigen.

Because we observed EGF mRNA in adherent wound cells (Fig. 2A) but not in cultured or cell line macrophages, it was probable that nonmacrophage wound cells were transcribing EGF. To provide direct evidence for TGF-α transcription by macrophages, we next examined highly purified cultured macrophages and macrophage cell lines. Thioglycollate-elicited mouse peritoneal macrophages were stimulated with LPS (*15*) for 6 hours or incubated in control medium, then purified by fluorescence-activated cell sorting (FACS) using the F4/80 monoclonal antibody to select only macrophages. The RNA phenotype was assayed by the RT-PCR method. TGF-α was expressed only by LPS-stimulated macrophages (Fig. 3A), whereas TGF-β was expressed constitutively, as previously reported (*4*). Cultured macrophages expressed IL-1α, G-CSF, GM-CSF, basic FGF, IGF-1, and PDGF-A but did not express nerve growth factor–β (NGF-β) or EGF transcripts (*12*). Therefore, FACS-purified macrophages were free of contaminating fibroblasts, which express NGF-β (*16*).

Mouse macrophage cell lines (P388D1, J774.1, RAW 264.7, and WEHI-3) stimulated with LPS also expressed TGF-α mRNA (*12*). The number of TGF-α transcripts in the P388D1 macrophage cell line was increased by treatment with LPS, and their single ~4.5-kb size, determined by RNA blot analysis (Fig. 3B), matches the size of the rat brain TGF-α transcript (*17*). TGF-α transcripts were detected within 3 hours of treatment of elicited macrophages with LPS, or after we loaded the macrophages with acetylated low-density lipoproteins (AcLDL), another stimulus relevant to tissue injury and wound healing (*18*) (Fig. 3C). Transcript levels remained high for 6 or 9 hours of LPS treatment but had disappeared by 48 hours.

These data suggest that macrophages are capable of synthesizing TGF-α mRNA transcripts under relevant circumstances. But transcription of growth factors does not always correspond qualitatively or quantitatively to translation (*4*). We next asked whether macrophages in culture translate the TGF-α mRNA. Antigen against TGF-α was secreted by LPS-stimulated elicited macrophages in a time-dependent manner, as determined by ELISA, at concentrations

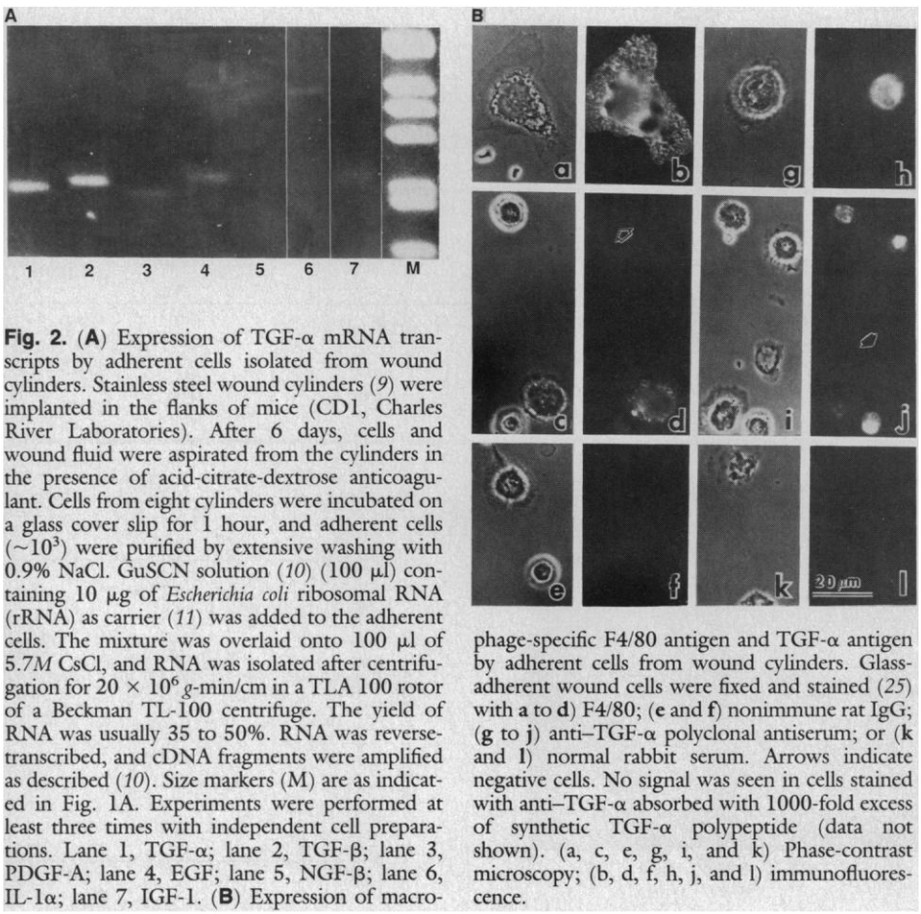

Fig. 2. (**A**) Expression of TGF-α mRNA transcripts by adherent cells isolated from wound cylinders. Stainless steel wound cylinders (*9*) were implanted in the flanks of mice (CD1, Charles River Laboratories). After 6 days, cells and wound fluid were aspirated from the cylinders in the presence of acid-citrate-dextrose anticoagulant. Cells from eight cylinders were incubated on a glass cover slip for 1 hour, and adherent cells (~10^3) were purified by extensive washing with 0.9% NaCl. GuSCN solution (*10*) (100 μl) containing 10 μg of *Escherichia coli* ribosomal RNA (rRNA) as carrier (*11*) was added to the adherent cells. The mixture was overlaid onto 100 μl of 5.7*M* CsCl, and RNA was isolated after centrifugation for 20 × 10^6 *g*-min/cm in a TLA 100 rotor of a Beckman TL-100 centrifuge. The yield of RNA was usually 35 to 50%. RNA was reverse-transcribed, and cDNA fragments were amplified as described (*10*). Size markers (M) are as indicated in Fig. 1A. Experiments were performed at least three times with independent cell preparations. Lane 1, TGF-α; lane 2, TGF-β; lane 3, PDGF-A; lane 4, EGF; lane 5, NGF-β; lane 6, IL-1α; lane 7, IGF-1. (**B**) Expression of macrophage-specific F4/80 antigen and TGF-α antigen by adherent cells from wound cylinders. Glass-adherent wound cells were fixed and stained (*25*) with **a** to **d**) F4/80; (**e** and **f**) nonimmune rat IgG; (**g** to **j**) anti–TGF-α polyclonal antiserum; or (**k** and **l**) normal rabbit serum. Arrows indicate negative cells. No signal was seen in cells stained with anti–TGF-α absorbed with 1000-fold excess of synthetic TGF-α polypeptide (data not shown). (a, c, e, g, i, and k) Phase-contrast microscopy; (b, d, f, h, j, and l) immunofluorescence.

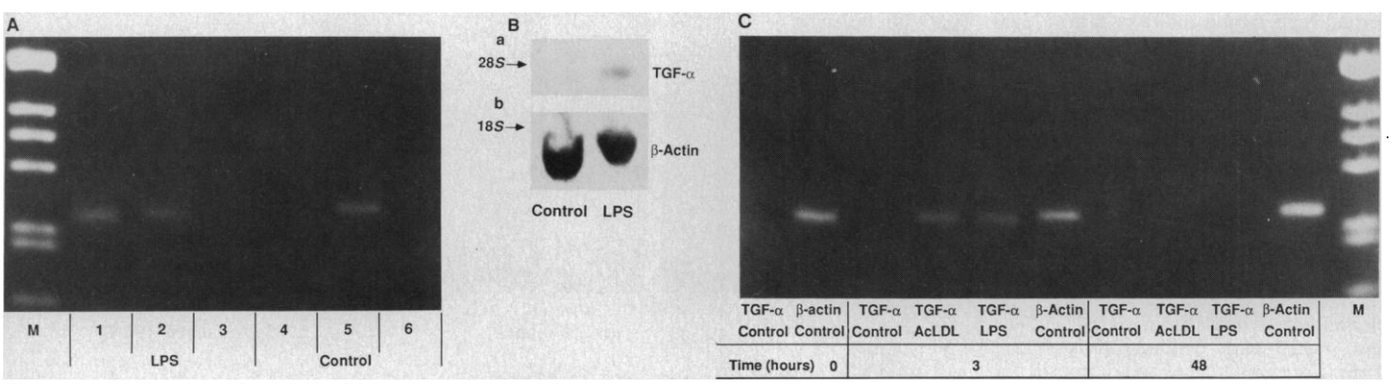

Fig. 3. (**A**) Expression of TGF-α mRNA transcripts by cultured macrophages stimulated with LPS. Adherent thioglycollate-elicited peritoneal exudate cells (*15*) were cultured for 6 hours in the presence of LPS (10 μg/ml) or in medium alone (control). Macrophages were selected by FACS as the brightest 10% of cells binding F4/80. RNA from these F4/80-positive cells was reverse-transcribed, and cDNA fragments specific for TGF-α and TGF-β were amplified as described in Fig. 1A. Lanes 1 and 4, TGF-α; lanes 2 and 5, TGF-β; lanes 3 and 6, NGF-β. (**B**) Expression of TGF-α mRNA transcripts by P388D1 macrophages (*26*) stimulated with LPS (*27*). Arrows indicate migration of 28*S* and 18*S* rRNA bands. (**C**) Expression of TGF-α mRNA transcripts by lipid-loaded or LPS-stimulated cultured macrophages in a time-dependent manner. TEPM were cultured in serum-free medium (control) or stimulated with LPS (10 μg/ml) or AcLDL (50 μg/ml, Biomedical Technology, Inc.) for 3 to 48 hours. The cDNA fragments were generated as described in Fig. 1A. A similar pattern of TGF-α expression was seen in TEPM treated for 6 or 9 hours with LPS (*12*).

similar to those found in wound fluid (Fig. 4A). The antigen detected in the ELISA was an authentic macrophage translation product, as demonstrated by immunoprecipitation of TGF-α of ~6 kD from biosynthetically labeled secreted macrophage proteins (Fig. 4A). At least 90% of the LPS-stimulated cultured macrophages actively synthesized TGF-α, as analyzed by immunocytochemistry, whereas few, if any, of the unstimulated cultured macrophages expressed intracellular TGF-α (Fig. 4B). Cultured P388D1 macrophages or elicited peritoneal macrophages were next stimulated with LPS for 48 hours in serum-free medium to determine production of mitogenic activity. Medium was concentrated by dialysis and lyophilization, fractionated by heparin-Sepharose affinity chromatography, and assayed for mitogenic stimulation of quiescent confluent Balb/c 3T3 fibroblasts (*19*). As described by Shing *et al.* (*20*), EGF does not bind to heparin-Sepharose, PDGF binds and elutes at low NaCl concentration, and basic FGF binds and elutes at high NaCl concentration. Mitogenic activity produced by P388D1 cells eluted under these three conditions. These peaks were pooled and assayed by radioreceptor assay to detect TGF-α/EGF or by dot immunoblot assay to detect PDGF, TGF-β, or basic FGF (Table 2). A major component of the total mitogenic protein and activity in macrophage-conditioned medium was TGF-α (Fig. 4A and Table 2). Similar elution profiles were seen for P388D1 cells and elicited macrophages, except that FGF was not secreted by elicited macrophages. We do not know if the FGF in conditioned medium of P388D1 cells was due to secretion or to a low incidence of cell death. The viability of both elicited peritoneal macrophages and P388D1 macrophages was greater than 98% in all cultures, but the intracellular pool of basic FGF in P388D1 was not quantified.

An essential process such as wound healing has redundant components. In vivo, TGF-α is thought to mediate epidermal regrowth, angiogenesis, and formation of granulation tissue (*8*). Macrophage-derived TGF-β and basic FGF are potentially angiogenic. Macrophage-derived IL-1, TGF-β, PDGF, and basic FGF could cause formation of granulation tissue. TGF-β may mediate epidermal regrowth (*21*), but this is likely to be an indirect function of its powerful chemoattraction for macrophages (*22*). Taken together, our data indicate that TGF-α delivered by macrophages may be a direct mediator of wound healing. The discovery that wound macrophages express multiple growth factors, and produce them under wound-healing conditions in vivo, will have important biological ramifications. The technology developed to detect transcripts from a single cell or a few cells should be applicable to many physiological questions.

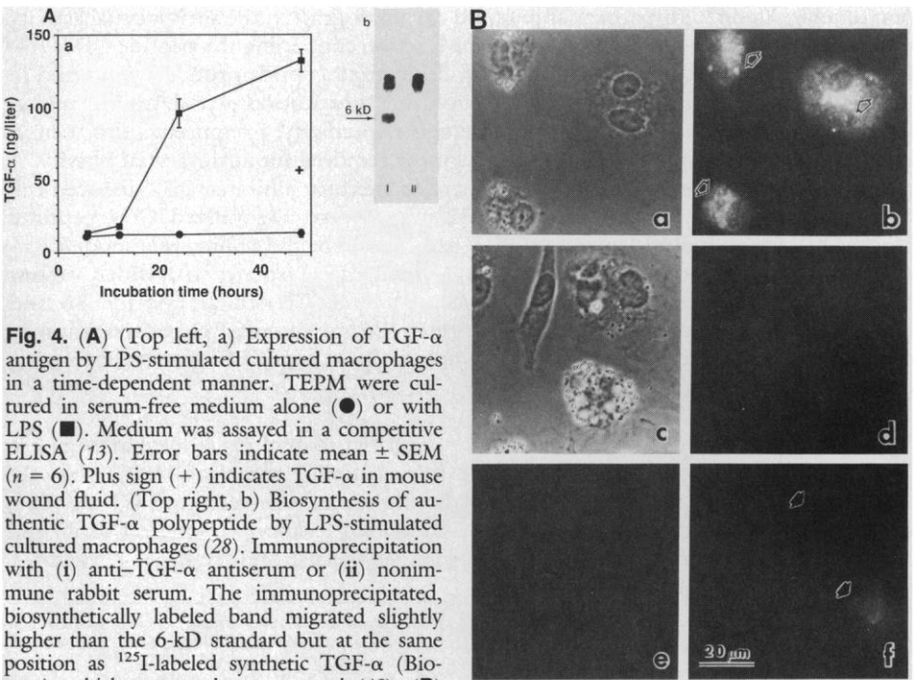

Fig. 4. (**A**) (Top left, a) Expression of TGF-α antigen by LPS-stimulated cultured macrophages in a time-dependent manner. TEPM were cultured in serum-free medium alone (●) or with LPS (■). Medium was assayed in a competitive ELISA (*13*). Error bars indicate mean ± SEM (n = 6). Plus sign (+) indicates TGF-α in mouse wound fluid. (Top right, b) Biosynthesis of authentic TGF-α polypeptide by LPS-stimulated cultured macrophages (*28*). Immunoprecipitation with (**i**) anti–TGF-α antiserum or (**ii**) nonimmune rabbit serum. The immunoprecipitated, biosynthetically labeled band migrated slightly higher than the 6-kD standard but at the same position as ^{125}I-labeled synthetic TGF-α (Biotope), which was used as a control (*12*). (**B**) Expression of intracellular TGF-α antigen by LPS-stimulated cultured macrophages. TEPM were stimulated for 24 hours with 10 μg/ml LPS or were cultured in serum-free medium, then fixed and stained (*25*). (**a** and **b**) Anti–TGF-α staining of LPS-stimulated TEPM; open arrows indicate perinuclear staining concentrated in the Golgi region; (**c** and **d**) anti–TGF-α staining of unstimulated TEPM; (**e**) staining of LPS-stimulated TEPM with nonimmune rabbit serum; (**f**) Staining of LPS-stimulated TEPM with anti–TGF-α absorbed with a 1000-fold excess of TGF-α polypeptide; arrows indicate diminished perinuclear staining compared to (b). (a and c) Phase-contrast microscopy; (b, d, e, and f) immunofluorescence.

REFERENCES AND NOTES

1. S. J. Leibovich, in *Soft and Hard Tissue Repair: Biological and Clinical Aspects*, T. K. Hunt, R. B. Heppenstall, E. Pines, D. Rovee, Eds. (Praeger, New York, 1984), pp. 329–356; D. R. Knighton *et al.*, *Science* **221**, 1283 (1983).
2. S. J. Leibovich and R. Ross, *Am. J. Pathol.* **78**, 71 (1975).
3. S. B. Mizel, *Immunol. Rev.* **63**, 51 (1982); K. Shimokado *et al.*, *Cell* **43**, 277 (1985); Y. Martinet *et al.*, *Nature* **319**, 158 (1986); A. Baird, P. Mormède, P. Böhlen, *Biochem. Biophys. Res. Commun.* **126**, 358 (1985); B. Thorens, J.-J. Mermod, P. Vassalli, *Cell* **48**, 671 (1987); C. J. Wiedermann *et al.*, *J. Immunol.* **137**, 3928 (1986); A. Rambaldi, D. C. Young, J. D. Griffin, *Blood* **69**, 1409 (1987); N. A. Nicola and D. Metcalf, in *Biochemistry of Macrophages* (Pitman, London, 1986), pp. 7–22.
4. R. K. Assoian *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6020 (1987).
5. S. J. Leibovich *et al.*, *Nature* **329**, 630 (1987).
6. M. Frater-Schröeder *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5277 (1987).
7. G. Carpenter, *Cell* **37**, 357 (1984).
8. A. B. Schreiber, M. E. Winkler, R. Derynck, *Science* **232**, 1250 (1986); G. S. Schultz *et al.*, *ibid.* **235**, 350 (1987); A. Buckley *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7340 (1985).
9. J. A. Schilling, B. U. Favata, M. Radakovich, *Surg. Gynecol. Obstet.* **96**, 143 (1953); M. J. Banda, D. R. Knighton, T. K. Hunt, Z. Werb, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7773 (1982).
10. RNA was purified by a modification of the guanidine thiocyanate (GuSCN)/CsCl gradient technique [J. M. Chirgwin, A. E. Przybyla, R. J. MacDonald, W. J. Rutter, *Biochemistry* **18**, 5294 (1979)] and reverse-transcribed by using 100 U of Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and 0.2 μg oligo(dT) as primer for 1 hour at 37°C [G. F. Gerard, *Focus (Bethesda Res. Lab.)* **9**, 5 (1987)]. One-tenth of the resulting cDNA was amplified with 1 U of *Thermus aquaticus* (Taq) polymerase (Perkin-Elmer Cetus) and primers (1 μ*M* each) during 60 to 90 cycles of polymerase chain reaction (PCR) [R. K. Saiki *et al.*, *Science* **239**, 487 (1988); R. K. Saiki *et al.*, *ibid.* **230**, 1350 (1985)] on a Perkin-Elmer Cetus PCR 1000 Thermocycler. Each cycle included denaturation at 93°C, reannealing of primer and fragment at 55°C, and primer extension at 72°C. Six microliters of the 50-μl PCR reaction mix were fractionated electrophoretically in a 3% NuSieve GTG/1% SeaKem ME agarose gel. Gels were run at 80 V until the bromphenol blue dye front had migrated 6 cm and were then stained with ethidium bromide.
11. D. A. Rappolee *et al.*, *J. Cell. Biochem.*, in press.
12. D. A. Rappolee *et al.*, data not shown.
13. E. T. Maggio, *Enzyme Immunoassay* (CRC Press, Boca Raton, FL, 1981). A competitive ELISA was developed for use with polyclonal antiserum to TGF-α (anti–TGF-α) and TGF-α synthetic polypeptide (Peninsula Laboratories) or with biologically active TGF-α (Biotope). Antigen was dried onto NUNC-F microtiter plates at *p*H 10 and washed with a solution of bovine serum albumin in phosphate-buffered saline (PBS). A dilution series of TGF-α (1 to 1000 pg) and a constant 1/5000 dilution of anti–TGF-α were used to generate a standard curve. Biotinylated goat antibody to rabbit immunoglobulin G (IgG) (HyClone) (anti-rabbit IgG) was used as a secondary antibody, and the ELISA amplification kit (BRL) was used for color development. As much as 10 ng of EGF (Collaborative Research) was negative in the assay. For assay, conditioned culture medium was collected at 6, 12, 24, and 48 hours, dialyzed against 0.2*M* acetate buffer, *p*H 4.5, and resuspended at 10× concentration.

14. The TGF-α/EGF radioreceptor assay was performed with a kit from Biomedical Technology, Inc., in accordance with the manufacturer's instructions.
15. Z. Werb and J. R. Chin, *J. Biol. Chem.* **258**, 10642 (1983); Z. Werb and J. R. Chin, *J. Exp. Med.* **158**, 1272 (1983).
16. K. Siminoski, J. Bernanke, C. Kay, R. A. Murphy, *Endocrinology* **118**, 1417 (1986); D. Clegg, L. Reichardt, D. A. Rappolee, Z. Werb, manuscript in preparation.
17. Oligonucleotide sequences are designed from the following cDNA sequences: β-actin, S. Alonso, A. Minty, Y. Bourlet, M. Buckingham, *J. Mol. Evol.* **23**, 11 (1986); TGF-β, D. C. Lee, T. M. Rose, N. R. Webb, G. J. Todaro, *Nature* **313**, 489 (1985); TGF-α, R. Derynck, J. A. Jarrett, E. Y. Chen, D. V. Goeddel, *J. Biol. Chem.* **261**, 4377 (1986); PDGF-A, C. Betsholtz *et al.*, *Nature* **320**, 695 (1986); EGF, A. Gray, T. J. Dull, A. Ullrich, *ibid.* **303**, 722 (1983); IL-1α, P. T. LoMedico *et al.*, *ibid.* **312**, 458 (1984); IGF-1, G. I. Bell *et al.*, *Nucleic Acids Res.* **20**, 7873 (1986). Whenever possible, the positions of the oligonucleotide primers are chosen to include a diagnostic restriction endonuclease site and an intron so that PCR fragments generated from any contaminating genomic DNA can be distinguished from those generated from the mRNA (*11*).
18. J. L. Goldstein, Y. K. Ho, S. K. Basu, M. S. Brown, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 333 (1979); M. S. Brown and J. L. Goldstein, *Nature* **316**, 680 (1985); R. Takemura and Z. Werb, *J. Exp. Med.* **159**, 167 (1984); R. Ross, *N. Engl. J. Med.* **314**, 488 (1986).
19. Mitogenesis was assayed by determining [^{3}H]thymidine uptake into quiescent confluent Balb/c 3T3 fibroblasts. Fetal calf serum (10%) was used as a positive control, and 10% RPMI 1640 was used as a negative control for the mitogenesis assay by the methods of W. Wharton, G. Y. Gillespie, S. W. Russell, W. J. Pledger, *J. Cell. Physiol.* **110**, 93 (1982).
20. Y. Shing *et al.*, *Science* **223**, 1296 (1984).
21. T. A. Mustoe *et al.*, *ibid.* **237**, 1333 (1987).
22. S. M. Wahl *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5788 (1987).
23. For the dot immunoblot assay [P. V. Talbot, R. L. Knobler, M. J. Buchmeier, *J. Immunol. Methods* **73**, 177 (1984)] basic FGF column fraction samples or 1 to 10,000 pg of basic FGF or acidic FGF (a gift of D. Gospodarowicz) were dotted onto nitrocellulose, dried, and blocked with 3% normal rabbit serum, then with 1/1000 dilution of a monoclonal antibody to basic FGF (a gift of D. Gospodarowicz), developed with biotinylated rabbit antibody to mouse IgG (Hyclone) diluted 1/1000, followed by alkaline phosphatase-labeled avidin (Vector) at 0.2 U/ml. For immunodot assays of PDGF, samples of macrophage-conditioned medium or 0.01 to 1.0 U of PDGF (Collaborative Research) were dotted onto nitrocellulose, dried, blocked with 3% normal goat serum, then incubated with 1/500 dilution of goat polyclonal antibody to PDGF (a gift of G. Grotendorst) and developed with biotinylated sheep antibody to goat IgG (Sigma) at 1/1,000, followed by alkaline phosphatase–labeled avidin (Vector), nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate (Sigma). The TGF-β immunodot assay was performed in a similar manner with anti-TGF-β (R&D Systems, Inc.).
24. D. Mark, unpublished results.
25. For immunofluorescence staining, adherent cells from wound cylinders or TEPM on 12-mm glass cover slips were fixed for 10 to 30 min in freshly prepared 2% paraformaldehyde, then washed in PBS containing 0.1% glycine. Cells to be stained with F4/80, a macrophage-specific rat monoclonal antibody [J. M. Austyn and S. Gordon, *Eur. J. Immunol.* **11**, 805 (1981)] were left unpermeabilized. Cells to be stained with anti–TGF-α (Peninsula Laboratories) were permeabilized with 0.25% Triton X-100 for 4 min. Nonspecific binding sites were then blocked with ovalbumin (1 mg/ml) for 30 min, followed by 5% skim milk powder for 30 min. Samples were then stained for 1 hour with F4/80 or nonimmune rat IgG at 20 μg/ml, or with anti–TGF-α or nonimmune rabbit serum at 1:100, diluted in 5% skim milk. After extensive washing, cells were then incubated with biotinylated F(ab′)$_2$ fragments from either anti-rat IgG or anti-rabbit IgG (Hy-Clone), diluted 1:100, washed, and stained with Texas red–labeled streptavidin (Amersham). Cells were observed with a 63× water-phase PlanNeofluor lens on a Zeiss Photomicroscope III and photographed on Tri-X film rated at 800 ASA for 60-second exposures.
26. P. Ralph, in *Mononuclear Phagocytes: Functional Aspects*, R. van Furth, Ed. (Nijhoff, The Hague, 1980), pp. 439–456; Z. Werb and J. R. Chin, *J. Cell Biol.* **97**, 1113 (1983).
27. RNA (20 μg) from LPS-stimulated or control P388D1 cells was fractionated on a 1% denaturing agarose gel, transferred to a nylon membrane, and cross-linked by ultraviolet irradiation for 3 min at 9 cm at 16 J/m^2 per second [G. M. Church and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1991 (1984)]. The blot was hybridized with a random-primed plasmid insert from mouse TGF-α cDNA (a gift of R. Derynck) and then rehybridized with a random-primed β-actin plasmid (a gift of M. Kirschner).
28. LPS-stimulated TEPM were radiolabeled with [^{35}S]cysteine (100 μCi/ml; specific activity, 1135 Ci/mmol) for 24 hours. Conditioned medium was processed as described (*13*) and was fractionated on a TSK 4000SW (30 cm) to 3000SW (60 cm) size exclusion column system equilibrated and run with a mobile phase of 0.1% trifluoroacetic acid–40% acetonitrile. The 3- to 20-kD fractions were immunoprecipitated with anti–TGF-α antiserum or nonimmune rabbit serum, separated by SDS–polyacrylamide gel electrophoresis on a 7 to 15% gradient gel under denaturing conditions, and then autoradiographed.
29. We thank K. S. Dwyer for implanting the wound cylinders, S. Gordon (University of Oxford) for the macrophage-specific rat monoclonal antibody and A. Wang, D. Gospodarowicz, G. Neufeld, P. Crocker, and D. Clegg for helpful comments. Supported by a contract from the Office of Health and Environmental Research, U.S. Department of Energy (DE-AC03-76-SF01012), NIH grants AR 32746 and GM 27345, and a University of California Regents Fellowship and University of California Patent Fund Award (to D.A.R.).

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Synthetic CD4 Peptide Derivatives That Inhibit HIV Infection and Cytopathicity

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Synthetic peptide segments of the CD4 molecule were tested for their ability to inhibit infection of CD4$^+$ cells by the human immunodeficiency virus (HIV) and to inhibit HIV-induced cell fusion. A peptide mixture composed of CD4(76–94), and synthesis side products, blocked HIV-induced cell fusion at a nominal concentration of 125 micromolar. Upon high-performance liquid chromatography, the antisyncytial activity of the peptide mixture was found not in the fraction containing the peptide CD4(76–94) itself, but in a side fraction containing derivatized peptide products generated in the automated synthesis. Derivatized deletion and substitution peptides in the region CD4(76–94) were used to demonstrate sequence specificity, a requirement for benzyl derivatization, and a core seven-residue fragment required for antisyncytial activity. A partially purified *S*-benzyl-CD4(83–94) peptide mixture inhibited HIV-induced cell fusion at a nominal concentration of ≤32 micromolar. Derivatized CD4 peptides blocked cell fusion induced by several HIV isolates and by the simian immunodeficiency virus, SIV, and blocked infection in vitro by four HIV-1 isolates with widely variant envelope gene sequences. Purified CD4(83–94) dibenzylated at cysteine 86 and glutamate 87 possessed antisyncytial activity at 125 micromolar. Derivatization may specifically alter the conformation of CD4 holoreceptor peptide fragments, increasing their antiviral efficacy.

LIGANDS THAT BIND TO, BUT DO NOT activate, biological receptors are called antagonists, and are used therapeutically as competitive inhibitors of ligand-receptor interactions (*1*). It should also be possible to use fragments of receptors as competitive inhibitors of ligand-receptor binding, provided that a small continuous region of a receptor binds the ligand and that this epitope, separated from the holoreceptor, has sufficient conformational stability to allow a thermodynamically favorable interaction with the ligand.

CD4 is a 55- to 58-kD glycoprotein originally characterized as a T lymphocyte differentiation antigen (*2*). It is implicated as an associative recognition element in major histocompatibility complex (MHC) class II–restricted immune responses (*3*) including the mixed leukocyte response (MLR) and antigen-specific MHC class II–restricted T

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