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Breast Cancer–Associated pS2 Protein: Synthesis and Secretion by Normal Stomach Mucosa

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The human pS2 gene is specifically expressed under estrogen transcriptional control in a subclass of estrogen receptor–containing human breast cancer cells. The pS2 gene encodes an 84–amino acid protein that is secreted after signal peptide cleavage. The distribution of pS2 protein in normal human tissues was studied with antibodies to pS2; pS2 was specifically expressed and secreted by mucosa cells of the normal stomach antrum and body of both female and male individuals. Moreover, no estrogen receptor could be detected in these cells, indicating that pS2 gene expression is estrogen-independent in the stomach. The function of the pS2 protein in the gastrointestinal tract is unknown. However, the pS2 protein is similar in sequence to a porcine pancreatic protein that has been shown to inhibit gastrointestinal motility and gastric secretion.

The human pS2 gene was discovered by differential screening of a cDNA library from breast cancer cells (MCF-7) grown in the presence of estrogen (*1*). Estrogen induction of pS2 mRNA synthesis corresponds to a primary transcriptional event (*2*), and the single-copy pS2 gene (*3*) encodes a cysteine-rich 84–amino acid polypeptide bearing characteristics of a secreted protein (*4*). Secretion of pS2 from MCF-7 cells was demonstrated by means of polyclonal antibodies directed against a synthetic peptide that corresponds to the carboxyl terminal half of the cDNA-deduced amino acid sequence (*5*). These antibodies and RNA blotting analysis have been used to show that the pS2 gene is specifically expressed in a subclass of estrogen receptor (ER)–containing breast cancers, and a preliminary survey of normal and tumoral human tissues and cells indicated that this expression was restricted to some primary breast cancers and their lymph node metastases (*6*).

Paraffin-embedded sections of endoscopic biopsies of normal stomach antrum and body were reacted with rabbit polyclonal antibodies directed against a synthetic peptide corresponding to the last 31 amino acids of pS2 (*5, 6*) and stained with a peroxidase-labeled second antibody. Material cross-reactive to pS2 was detected in mucosa cells of the superficial epithelium and crypts of both antrum (Fig. 1D, left side) and body (not shown) of the stomach. No staining was observed in body glands. Staining was specific for pS2, as it was suppressed by the presence of the synthetic peptide used to raise the antibodies (Fig. 1E). Similar results were obtained with a monoclonal antibody directed against a synthetic peptide corresponding to the last 28 amino acids of the pS2 protein. The corresponding staining of pS2 protein–positive cells of a ductal breast carcinoma is shown for comparison (Fig. 1, A and B). As in breast tumor cells (*6*), pS2 staining in stomach mucosa cells was predominantly cytoplasmic with an uneven distribution and often a perinuclear localization (Fig. 1D, right side), which may correspond to the Golgi apparatus. The pS2-specific staining was observed in both male and female stomach mucosa. However, no significant staining was observed in a variety of normal human specimens (colon, esophagus, gallbladder, pancreas, liver, thyroid and parathyroid glands, skin, lymph nodes, lung, brain, bladder, prostate, kidney, adrenal gland, testis, placenta, endometrium, ovary, and pituitary gland). Weak staining was observed in salivary glands.

When monoclonal antibodies directed against the human ER were used, the presence of nuclear ER was readily revealed in frozen sections of pS2 protein–positive breast carcinoma (*6*) (Fig. 1C). In marked contrast, no ER could be detected in frozen sections of stomach mucosa (Fig. 1F).

These results suggest that pS2 protein is synthesized by stomach mucosa cells and could possibly be secreted into the gastric fluid. Portions of stomach fluid obtained from fasting male and female individuals were acetone-precipitated, separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose, and reacted with the monoclonal antibody to pS2. In all cases immunoreactive material with an apparent size of approximately 7 kD, similar to that of the breast cancer pS2 protein (*5*), was readily revealed (Fig. 2, lanes 1 and 2). The presence of the synthetic peptide used for generating the monoclonal antibody eliminated staining (Fig. 2, lanes 3 and 4). The migration of the presumptive pS2 protein present in stomach fluid was then directly compared after extensive purification with that of pS2 protein purified from the culture medium of the breast cancer cell line MCF-7. Both proteins migrated identically and reacted similarly with the monoclonal antibody (Fig. 2, lanes 5 and 6, MCF-7 and gastric pS2 proteins, respectively). When pure pS2 protein prepared from MCF-7 cell culture medium was used as a standard, an average of 70 ng of pS2 protein per milliliter

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of gastric fluid was found (six samples were analyzed, and the limit values were 30 and 100 ng/ml).

To confirm the conclusion that the same pS2 protein is synthesized in stomach mucosa cells and in estrogen-dependent breast carcinoma cells, the structure of stomach pS2 mRNA was investigated by means of specific probes derived from the previously cloned pS2 cDNA (*4, 6*) and pS2 gene (*3*). Total RNA extracted from stomach antrum specimens was first analyzed by RNA blotting with a nick-translated pS2 cDNA probe and the 36B4 cDNA probe, which corresponds to a ubiquitous human mRNA (*1, 6*). In all cases an RNA comigrating with the pS2 mRNA present in MCF-7 cells, approximately 600 nucleotides (nt) in length, was detected (Fig. 3d). The relative amount of pS2 mRNA was at least as high, if not higher, in stomach antrum as in MCF-7 cells cultured in the presence of estradiol. The fine structure of the stomach pS2 mRNA was then compared with that of its MCF-7 counterpart by means of three overlapping antisense RNA probes (Fig. 3a). Probe A is approximately 720 nt in length and contains the sequence corresponding to exon 1 of the pS2 gene, whereas probe B (207 nt) corresponds to the remaining part of the pS2 coding region; probe C encompasses all three pS2 exons except the last 10 bp of the exon 3 untranslated region. In all three cases, the same protected bands were observed by using either 10 μg of total stomach RNA or 30 μg of estrogen-treated MCF-7 cell RNA (Fig. 3, b and c). Moreover, the size of the protected bands was as expected from the DNA sequence of the pS2 gene and no unexpected bands were seen, indicating that not only the length, but also the sequence, of the pS2 mRNA is the same in MCF-7 and stomach mucosa cells. Thus, the primary transcript of the single-copy pS2 gene appears to be identically spliced in these two cell types, yielding the same mRNA and presumably the same protein. That the same protein is secreted in the stomach and in the culture medium of MCF-7 cells has been confirmed by amino acid sequencing of the pS2 proteins purified from either source (*7*).

The present results demonstrate that the same pS2 protein whose synthesis is specifically induced by estrogens in a subclass of breast cancer cells is also secreted in normal stomach mucosa cells of both males and females. A previous (*4*), as well as a more recent, search in the protein sequence databases indicates that the pS2 protein is a new protein with no significant homology with any other known human protein. However, the second search revealed an interesting similarity between the sequence of the pS2 protein and that of a secreted porcine pancreatic protein, pancreatic spasmolytic polypeptide (PSP) (*8–11*) (Fig. 4). The same similarity has been noted by Masiakowski

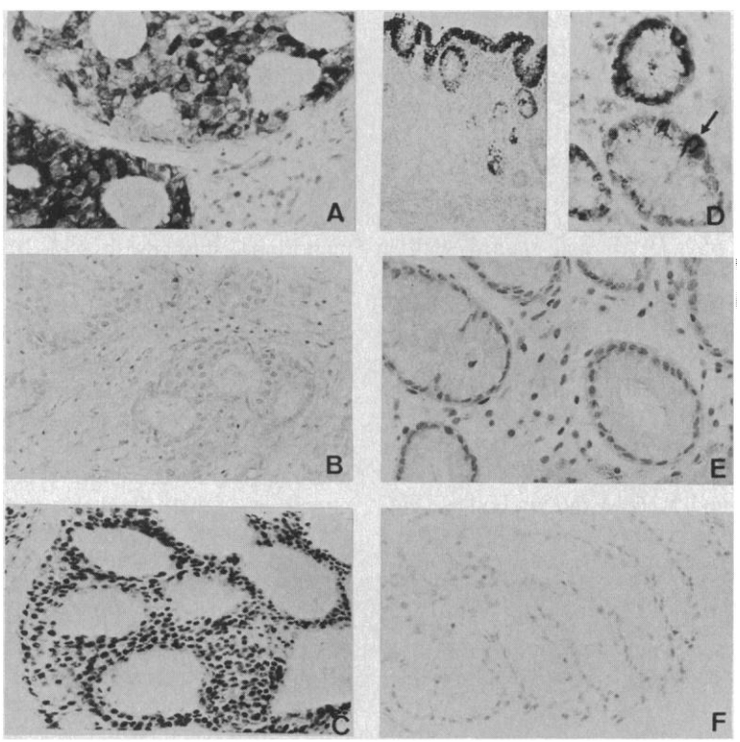

Fig. 1. Indirect immunoperoxidase staining of the pS2 protein in paraffin sections and of ER in frozen sections of stomach antrum mucosa and breast cancer. (**A**) The pS2-positive tumor cells of a breast ductal carcinoma. (**B**) Disappearance of pS2 staining when a section similar to that shown in (A) was stained in the presence of pS2 peptide (15 ng/ml) used to raise the antibodies. (**C**) ER-positive tumor cells in frozen sections of the same ductal carcinoma. (**D**) The pS2-positive cells in normal stomach antrum. (Left panel) In this section, which includes the whole thickness of the mucosa, cells of the superficial epithelium are heavily stained, whereas crypt mucous cells are more irregularly stained; (right panel) a higher magnification, showing that staining is uneven and often perinuclear (arrow). (**E**) Same material as in (D) stained in the presence of the pS2 peptide used to raise the antibodies. (**F**) A frozen section of the same material as in (D) was stained with the same ER monoclonal antibodies used (C); no ER-positive cells could be detected. All immunoreactions and peroxidase staining (peroxidase-antiperoxidase) were as described in (*6*). Magnifications: (A and B) ×500, (C and F) ×300, (D) ×125 (left) and ×700 (right), and (E) ×700.

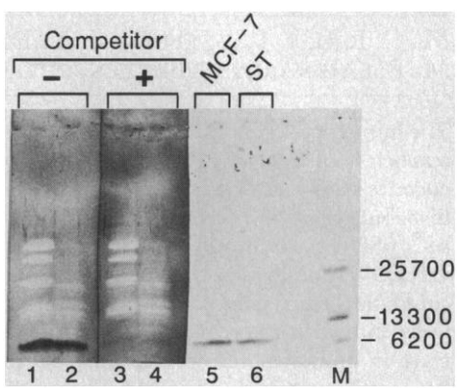

Fig. 2. Presence of pS2 immunoreactive material in gastric fluid. Two portions of stomach fluid obtained from either a fasting male (lanes 1 and 3) or a fasting female (lanes 2 and 4) individual were neutralized with bicarbonate buffer, *p*H 9.6, and acetone-precipitated overnight at −20°C. After dissolution of the pellet in electrophoresis sample buffer, aliquots corresponding to 0.4 ml of gastric fluid were separated by electrophoresis on an 18% SDS-polyacrylamide gel (SDS-PAGE) and electroblotted to nitrocellulose. After preincubation for 30 min in phosphate-buffered saline (PBS), *p*H 7.2, containing 3% bovine serum albumin at room temperature, proteins were reacted overnight at 4°C with a mouse monoclonal antibody (*7*) directed against a synthetic peptide corresponding to the last 28 amino acids of the pS2 protein (*5, 6*). After washing with PBS, *p*H 7.4, containing 0.1% Triton, the filter was reacted with a goat antiserum to mouse immunoglobulin G conjugated with peroxidase. After washing as above, the pS2 immunoreactive material was revealed by staining with dianisidine in the presence of H_2O_2. In lanes 3 and 4, the immunoreaction was carried out in the presence of the synthetic peptide used for raising the monoclonal antibody (~30 ng/ml). The acetone-precipitated protein was also purified from the culture medium of MCF-7 cells grown in the presence of estradiol (lane 5) or from stomach fluid (lane 6), by means of two high-performance liquid chromatography steps (G3000 sizing chromatography followed by RP300 hydrophobic chromatography). In both cases, aliquots of approximately 20 ng of purified pS2 were analyzed by protein immunoblotting as described above. Abbreviations: M, molecular size markers (in daltons); and ST, stomach sample.

(*12*) and by Thim (*13a*) and Baker (*13b*). There is a significant homology between the sequence of the pS2 protein starting at amino acid 31 and ending at its carboxyl end (amino acid 84) and the two homologous domains of PSP (Fig. 4). In pS2 protein the homologous region is encoded entirely by exon 2 and it appears therefore that the present-day PSP gene may have evolved by duplication from a pS2-like ancestor gene. It is unknown whether the human genome also contains a counterpart of the porcine PSP gene, but no RNA cross-hybridizing with the human pS2 cDNA has so far been detected in human pancreas and no pS2 immunoreactive material was seen in the same organ (*7*).

Previous studies have demonstrated that pS2 gene expression is under strict estrogen control in MCF-7 cells where its induction by estradiol is a primary transcriptional event (*2*). Moreover, an estrogen-responsive element has been identified upstream of the pS2 gene RNA startsite, which can act as an estrogen-inducible enhancer on heterologous promoter elements in HeLa cells expressing the human ER (*14*). In stomach mucosa cells of both males and females, the pS2 gene is expressed in the absence of this receptor (Fig. 1) and at a level apparently higher than in MCF-7 cells (Fig. 3). Therefore, the promoter region of the pS2 gene must be complex with different elements being responsible for specific control of transcription in breast cancer cells and in stomach mucosa cells.

The function of the pS2 protein in breast cancer cells, if any, is unknown. It could correspond to a growth factor (*4*), but this hypothesis has not yet been tested. Neither pS2 protein nor pS2 mRNA have been found in significant amounts in normal adult breast tissue of pre- or post-menopausal women (*6*), in breast tissue late in pregnancy or during lactation, or in human milk (*7*). Further, although the pS2 gene was readily detected in primates and its expression demonstrated in their stomach mucosa cells, no DNA or RNA sequences cross-hybridizing with the pS2 gene or pS2 protein immunoreactive material were found in mouse or pig stomach and pancreas (*7*). The function of the pS2 protein secreted in human stomach is also unknown. Could it be a gastric hormone that regulates gastric acid secretion, gastrointestinal motility, and growth of the gastrointestinal mucosa, or an enzyme inhibitor or cofactor? The homology between the pS2 protein and the porcine pancreatic protein PSP that inhibits gastrointestinal motility and gastric secretion in laboratory animals after parenteral as well as oral administration (*10, 15*) may suggest that the pS2 protein could play a similar role in humans. Overproduction of the pS2 protein in appropriate vectors and hosts will permit the physiological function of this new human gastric protein to be investigated.

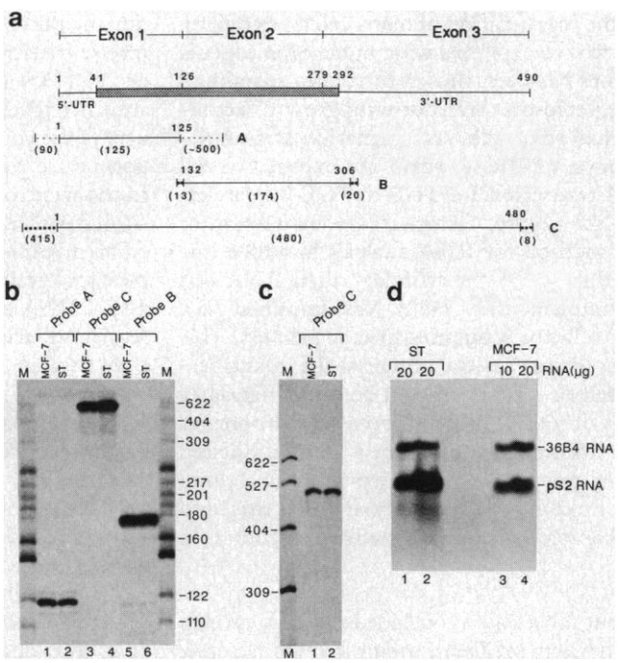

Fig. 3. Characterization of pS2 mRNA in normal stomach antrum. (**a**, **b**, and **c**) Schematic representation of the antisense RNA probes and autoradiograms of ribonuclease (RNase) mapping experiments. Total RNA extracted from stomach antrum specimens and MCF-7 cells was characterized by RNase mapping (*16, 17*) in (b) and (c) by using the antisense RNA probes represented schematically in (a). The genomic DNA fragment corresponding to probe A corresponds to exon 1 (125 bp) of the pS2 gene preceded by 90 bp upstream of the capsite and followed by about 500 bp of the first intron [0.7-kb B3 to B4 Bam HI fragment of the pS2 gene (*3*)]. This fragment was cloned into the Eco RI site of the polylinker of pBS M13+ (Stratagene). The cDNA (*4*) fragment corresponding to probe B corresponds to exon 2 from position 132 to the end and includes the first 27 bases of exon 3. This fragment was inserted between the Sma I–Pst I sites of pGEM1 (Promega Biotec). Probe C corresponds to the complete amino acid coding sequence of the pS2 cDNA (bases 41 to 292) and most of the 3′-untranslated region (*4*); this fragment was cloned between the Eco RI–Bam HI sites of pGEM1. Antisense RNA probes were prepared from the linearized plasmids by using [α-^{32}P]cytidine 5′-triphosphate and either SP6 (probes B and C) or T7 (probe A) RNA polymerases, according to the suppliers of pBS M13+ and pGEM1. Dotted lines in probes B and C represent the sequences of the vector transcribed together with the antisense RNA. Hybridization and RNase mapping were carried out (*16, 17*) by using 8% (b) or 4% (c) polyacrylamide-urea sequencing gels. The autoradiograms in (b) (c) correspond to the RNase mapping of the following RNA. Lanes 1, 3, and 5: 30 μg of total MCF-7 cell RNA; and lanes 2, 4, and 6: 10 μg of total RNA from normal stomach antrum (ST, samples from nine individuals were pooled). M indicates 5′ end-labeled Msp I fragments of pBR322. (**d**) RNA blot analysis of total RNA (as indicated) from normal ST (lanes 1 and 2 correspond to two different individuals) or MCF-7 cells grown in the presence of estradiol by using the diazobenzyloxymethyl paper technique and nick-translated pS2 and 36B4 internal control cDNA probes (*6*).

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1                                                          60
QKPAACRCSRQDPKNRVNCGFPGITSDQCFTSGCCFDSQVPGVPWCFSPLPAQES--EECVM   PSP
      **   ** * ******** **   ***** * ****** *        ***
      CT-VAPRERQNCGFPGVTPSQCANKGCCFDDTVRGVPWCFYPNTIDVPPEEECEF   pS2
31      * *** ***** *  ** * *** ***  ******* ***              84
        QVKARKNSGYPGICPEDCAARNCCFSDTIPEVPWCFFPMSVEDCHY           PSP
61                                                        106
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Fig. 4. Comparison of the amino acid sequence of the pS2 protein from amino acids 31 to 84 (*4*) with the complete sequence of the porcine PSP (*11*). The internal duplication of the PSP protein and the amino acid homology between the pS2 sequence and those of the two domains of the PSP protein are shown. The stars and the dots indicate amino acid identity and conservative changes, respectively, between the pS2 protein and the two PSP domains.

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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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