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Identification of Extractable Growth Factors From Small Intestinal Submucosa

Sherry L. Voytik-Harbin,* Andrew O. Brightman, Meredith R. Kraine, Beverly Waisner, and Stephen F. Badylak

Hillenbrand Biomedical Engineering Center, Purdue University, West Lafayette, Indiana 47907

Abstract When implanted as a biomaterial for tissue replacement, selected submucosal layers of porcine small intestine induce site-specific tissue remodeling. Small intestinal submucosa (SIS), as isolated, is primarily an acellular extracellular matrix material. In an attempt to discover the components of small intestinal submucosa which are able to induce this tissue remodeling, the material was extracted and extracts were tested for the ability to stimulate Swiss 3T3 fibroblasts to synthesize DNA and proliferate. Each of the four different extracts of small intestinal submucosa had measurable cell-stimulating activity when analyzed in both a whole cell proliferation assay (alamarBlue dye reduction) and a DNA synthesis assay ([^{3}H]-thymidine incorporation). Proteins extracted from SIS with 2 M urea induced activity profiles in the two assays which were very similar to the activity profiles of basic fibroblast growth factor (FGF-2) in the assays. As well, the changes in cell morphology in response to the extracted proteins mimicked the changes induced by FGF-2. Neutralization experiments with specific antibodies to this growth factor confirmed the presence of FGF-2 and indicated that it was responsible for 60% of the fibroblast-stimulating activity of the urea extract of small intestinal submucosa. Western blot analysis with a monoclonal antibody specific for FGF-2 detected a reactive doublet at approximately 19 kDa and further confirmed the presence of FGF-2. Cell stimulating activity of proteins extracted from SIS with 4 M guanidine was neutralized by an antibody specific for transforming growth factor β (TGFβ). Changes in the morphology of the fibroblasts exposed to this extract were nearly identical to changes induced by TGFβ. Although no reactive protein band was detected at 25 kDa in nonreduced western blot analysis, several bands were reactive at higher molecular weight. The identity of this TGFβ-related component of small intestinal submucosa is unknown. Identification of FGF-2 and TGFβ-related activities in SIS, two growth factors known to significantly affect critical processes of tissue development and differentiation, provides the opportunity to further elucidate the mechanisms by which this extracellular matrix biomaterial modulates wound healing and tissue remodeling. J. Cell. Biochem. 67:478–491, 1997.

Key words: growth factors; biomaterial; cell proliferation; extracellular matrix; tissue repair

The process of tissue regeneration, as opposed to scar formation, in response to tissue injury can be enhanced by the implantation of various biomaterials [Bell, 1995; Langer et al., 1995]. The emerging field of tissue engineering has focused on the development of naturally occurring and/or synthetic materials for tissue replacement or augmentation of wound repair [Hubbell, 1995]. The submucosa along with the adjacent connective tissue layer of mammalian small intestine has proven to be an excellent natural biomaterial for wound repair and tissue regeneration [Badylak, 1993].

Small intestinal submucosa (SIS), consisting primarily of extracellular matrix material (ECM), is prepared by mechanically removing selected portions of the mucosa and the external muscle layers and then lysing resident cells with hypotonic washes. Numerous studies have shown that this biomaterial is capable of inducing host tissue proliferation, remodeling, and

Abbreviations: ECL, enhanced chemiluminescense; ECM, extracellular matrix; EGF, epidermal growth factor; FGF-2, basic fibroblast growth factor; GF, growth factor; GFU, growth factor units; HRP, horseradish peroxidase; NNCS, neonatal calf serum; PDGF, platelet derived growth factor; PVDF, polyvinylidene difluoride; SIS, small intestinal submucosa; TGF, transforming growth factor.

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Meredith R. Kraine's current address is Dept. of Microbiology and Immunology, University of North Carolina, Chapel Hill, NC 27599.

*Correspondence to: Sherry L. Voytik-Harbin, Hillenbrand Biomedical Engineering Center, Purdue University, West Lafayette, IN 47907.

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regeneration of tissue structures following implantation in a number of in vivo microenvironments including lower urinary tract [Knapp et al., 1994], body wall [Prevel et al., 1995], tendon [Badylak et al., 1995], ligament [Aiken et al., 1994], blood vessels [Lantz et al., 1993], and bone [Voytik-Harbin, unpublished data]. Upon implantation, cellular infiltration and rapid neovascularization are observed and the SIS extracellular matrix material is remodeled into host replacement tissue with site-specific structural and functional properties [Badylak, 1993].

The normal process of tissue repair, whether regenerative or scar-forming, is characterized by a complex, multicomponent cascade of degradative and biosynthetic processes orchestrated by underlying cell-cell and cell-ECM interactions [Howell, 1996]. These processes involve and/or are directed by a variety of cell types, including monocyte/macrophages, fibroblasts and capillary endothelial cells. Secreted, circulating, and extracellular matrix-bound growth factors (GF) work in concert to regulate cell migration, proliferation, and differentiation throughout the repair process. PDGF, EGF, TGFα, TGFβ, and FGF-2 [Pierce et al., 1991; Schultz et al., 1991; Marikovsky et al., 1993; ten Dijke and Iwata, 1989; Werner et al., 1992; Greenhalgh et al., 1990] are just a few in an ever increasing list.

The events of tissue repair are similar to those of tissue development which occurs during embryonic and fetal growth and are likewise mediated through interactions between cells, extracellular matrix molecules, and GF. Direct interactions between extracellular matrix components and cells are known to mediate such processes as migration, proliferation, and differentiation during development [Roskelly et al., 1995]. However, the role of the extracellular matrix in tissue repair has been less well-studied [Yannas, 1994; Reddi, 1994; Hellman et al., 1994]. Studies of developmental processes have demonstrated that GF can regulate the synthesis and deposition of extracellular matrix components and in turn, these synthesized ECM components regulate the availability and activity of the GF. Thus it is likely that GF, in conjunction with the ECM, play a critical role in the remodeling processes of tissue repair.

To identify those growth factors which could be responsible for SIS-induced tissue regeneration, we have extracted this biomaterial and have conducted initial analyses of the components. The tissue was extracted with four different aqueous solvents and the extracts were evaluated for their effects on Swiss 3T3 fibroblasts. Two in vitro assays were used in parallel for the detection of factors capable of stimulating either whole cell proliferation or DNA synthesis. Specific antibodies directed against FGF-2 and TGFβ were used to identify the major fibroblast-stimulating factors extractable from SIS. This represents the first demonstration of GF isolated from the submucosal tissue of the intestine.

MATERIALS AND METHODS

Materials

alamarBlue was obtained from Alamar Bioscience Inc. (Sacramento, CA). [^{3}H]-thymidine (64.0 Ci/mmol) and enhanced chemiluminescence (ECL) reagents were purchased from Amersham Life Science Inc (Arlington Heights, IL). Bovine recombinant FGF-2 was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Purified porcine PDGF, recombinant human EGF, porcine TGFβ1 and β2, pan-specific TGFβ-neutralizing antibody and FGF-2-neutralizing antibody were purchased from R&D Systems (Minneapolis, MN). Recombinant human TGFβ3 standard was from Calbiochem (Cambridge, MA). Purified recombinant human FGF-2 and monoclonal antibody to FGF-2 were generously provided by Dr. Brad Olwin, Purdue University.

Cells

Swiss 3T3 mouse fibroblasts were obtained from American TypeCulture Collection (Rockville, MD). Cells were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, 2 mM glutamine, 1.5 g/l $NaHCO_3$, 100 U/ml penicillin, 100 ug/ml streptomycin, and 10% neonatal calf serum (NNCS). Medium components and serum were purchased from Sigma Chemical Co. (St. Louis, MO). Cells were grown in 75 cm^2 culture flasks and maintained in a humidified atmosphere of 5% CO_2 at 37°C. Subcultures were established every 3–4 days to prevent cells from exceeding 75% confluency. Cells representing limited passage numbers 9–13 were used for all assays.

Small Intestinal Submucosa

Small intestinal submucosa (SIS) was prepared from porcine intestine obtained from a local meat processing plant. Intestine was

rinsed free of contents, everted, and the superficial layers of the mucosa were removed by mechanical delamination. The tissue was reverted to its original orientation and the external muscle layer removed. The prepared SIS tube was split open longitudinally and rinsed extensively in water to lyse any cells associated with the matrix and to eliminate cell degradation products. Immediately after rinsing, SIS was frozen in liquid nitrogen and stored at −80°C. Frozen tissue was sliced into 1 cm cubes, pulverized under liquid nitrogen with an industrial blender, and stored at −80°C prior to use.

Extract Preparation

Extraction buffers used for these studies included 4 M guanidine, 2 M urea, 2 M $MgCl_2$, and 2 M NaCl, each prepared in 50 mM Tris-HCl, pH 7.4. SIS powder was suspended in extraction buffers (25% w/v) containing protease inhibitors (phenylmethyl sulfonyl fluoride, N-ethylmaleimide, and benzamidine each at 1 mM) and vigorously stirred for 24 h at 4°C. The extraction mixture was then centrifuged at 12,000*g* for 30 min at 4°C and the supernatant collected. The insoluble material was washed briefly in the extraction buffer, centrifuged, and the wash combined with the original supernatant. The supernatant was dialyzed extensively in Spectrapor tubing (MWCO 3500, Spectrum Medical Industries, Houston, TX) against 30 volumes of deionized water (nine changes over 72 h). The dialysate was centrifuged at 12,000*g* to remove any insoluble material and the supernatant either was used immediately or lyophilized for long-term storage.

Guanidine extract of bovine bone was prepared using the same procedure as for SIS except that 10 mM EDTA was included in the extraction buffer. The starting tissue was demineralized bone powder made from fresh bovine tibia as described [Ogawa and Seyedin, 1991].

Partial Purification of a TGFβ-Related Protein

Lyophilized guanidine extract of SIS was dissolved in 4 M guanidine/Tris pH 7.8, and proteins were loaded and separated on a 2.5 × 48 cm column of Sephacryl S300HR (Pharmacia Biotech, Gaithersburg, MD) at a flow of 0.6 ml/min and 9 ml fractions were collected in siliconized tubes. Fractions with anti-TGFβ neutralizable activity were pooled, dialysed against water and lyophilized.

alamarBlue Assay

alamarBlue is a metabolic (redox) indicator dye reduced by reactions of normal cellular metabolism and provides an indirect measure of viable cell number. This assay has been recently described [Voytik-Harbin et al., 1997] and is presented here in brief. 200 µl of Swiss 3T3 fibroblasts (8,000 cells/ml) in DMEM containing 1% NNCS were seeded into 96-well plates. After incubation for 24 h, standards and test substances were added to each well (50 µl). Following incubation for an additional 72 h, each well was examined microscopically to observe viability, number, and morphology of the cells. The medium was removed and fresh medium containing 1% NNCS and 10% alamarBlue was added to each well. After 18–20 h, dye reduction was monitored spectrofluorometrically using a LS-50B Luminescence Spectrometer (Perkin-Elmer, Oak Brook, IL) with excitation and emission wavelengths of 560 nm and 590 nm, respectively. All samples were assayed in triplicate. Background fluorescence measurements were determined from wells containing dye reagent in culture medium but no cells. The mean and standard deviation for all fluorescence measurements were calculated and subsequently corrected for background. One growth factor unit, GFU, was defined as half of the maximal response to serum above unstimulated background.

[^{3}H]-Thymidine Incorporation Assay

Mitogenic activity was quantitated by measuring incorporation of [^{3}H]-thymidine during DNA synthesis. 200 µl of Swiss 3T3 fibroblasts (14,000 cells/ml) in DMEM containing 10% NNCS were seeded into 96-well plates. The cells were grown to confluency (approximately 72 h) in a 5% CO_2 incubator at 37°C, at which time the medium was changed to DMEM containing 2% NNCS. Following incubation for 24 hours, standards or test substances (50 µl) were added to each well. After an additional 24 h incubation, 1 µCi of [^{3}H]-thymidine in 20 µl PBS, pH 7.4, was added to each well. Four h later, the medium was removed and the cells were treated with 0.1% trypsin (100 µl) by incubation at 37°C for at least 10 min. The cells were harvested onto glass fiber filters using a 290 PHD Cell Harvester (Cambridge Technology Inc., Watertown, MA), washed repeatedly with water, and then rinsed with 70% ethanol.

The filters were air dried and placed in 5 ml EcoLite (ICN, Pharmaceuticals, Costa Mesa, CA) liquid scintillant for radioactivity determination using a 1900TR Liquid Scintillation Analyzer (Packard, Downers Grove, IL). All standards and test substances were assayed in triplicate and the mean and standard deviations calculated. One GFU was defined as half of the maximal response to serum above unstimulated background.

Neutralization With Specific Antibodies

Dose-response curves for TGFβ1, FGF-2, PDGF, EGF, and SIS extracts were generated in both [^{3}H]-thymidine and alamarBlue assays. A concentration of each factor or extract which was near the top of the linear response in the assays was used for neutralization studies. Extracts and purified GFs were incubated for 1 h at 37°C in the absence and presence of specific neutralizing antibodies at antibody concentrations ranging from 0.5 µg/ml to 200 µg/ml. Control assays with antibody alone were tested at all relevant concentrations with no effects on either assay. Bioactivity of the neutralized samples was then analyzed in both the [^{3}H]-thymidine and alamarBlue assays.

Immunodetection of FGF-2 and TGF β-Related Protein

Extracts or fractions were mixed with sample buffer and separated on 4–20% gradient or 16.5% SDS-PAGE. The proteins were transferred to PVDF paper in 10 mM CAPS buffer, 10% methanol, 1.3 mM SDS in a wet transfer system at 500 mA for 3 to 4 h at 4°C. Blots were blocked with 5% dry milk, 0.05% Tween-20 in PBS for 2 h at room temperature or overnight at 4°C. Primary antibodies were diluted in antibody buffer [1% BSA, 0.05% Tween-20 in PBS] and were incubated with the blots for 2 h at 37°C. The blots were washed with 0.05% Tween-20 in PBS. Secondary antibodies coupled to peroxidase (HRP) were diluted in antibody buffer and incubated with blots for 1 h at room temperature. After final washing, the blots were incubated with ECL reagents and exposed to hyperfilm-ECL as directed by the manufacturer (Amersham Life Science Inc.). Specific dilutions for antibodies are described in the figure legends.

RESULTS

Growth Factor Activity Extracted From Small Intestinal Submucosa

Several chaotropic aqueous solvents were selected to extract potential GF from SIS. Extraction of powdered SIS tissue with high concentrations of guanidine, urea, NaCl, or $MgCl_2$ under neutral buffer conditions was effective in removing GF activity. Dose-response curves comparing purchased GFs or the different extracts in two in vitro bioassays demonstrated the utility of multiple assays and multiple extractions. The two assays provided distinct and complementary information. The more traditional mitogen assay ([^{3}H]-thymidine incorporation) measured the stimulation of DNA synthesis of a confluent, quiescent monolayer of fibroblasts. In complement, the whole cell proliferation assay (alamarBlue dye reduction) measured indirectly the increase in cell number from a low density, quiescent population of fibroblasts. Both bioassays were standardized using neonatal calf serum in standard curves and results were expressed as relative growth factor units, GFU (Fig. 1).

The responses of several commercially available GFs were evaluated and each GF gave a characteristic profile of activity in the two assays (Fig. 2). However, a general trend of higher stimulation of DNA synthesis than whole cell proliferation relative to the serum standards was seen for these purified GFs. Porcine TGFβ1 stimulated a moderate increase in both DNA synthesis and whole cell proliferation of fibroblasts over a broad range of TGFβ1 concentrations (Fig. 2A). PDGF induced a high level of DNA synthesis with little or no detectable whole cell proliferation (Fig. 2C). Purified EGF showed a dose-dependent increase in both DNA synthesis and whole cell proliferation of fibroblasts with maximal stimulation at 5 ng/ml in both assays (Fig. 2D). FGF-2 was unique among the GF tested in that its effect upon fibroblasts produced negative values in the alamarBlue assay while inducing a high level of stimulation of DNA synthesis (Fig. 2B). In both assays with FGF-2, most cells were rounded-up and had a high nuclear to cytoplasmic ratio characteristic of highly stimulated cells which are unable to complete the cell cycle. This state of the cells apparently decreases the cellular metabolism necessary for the alamarBlue dye reduction

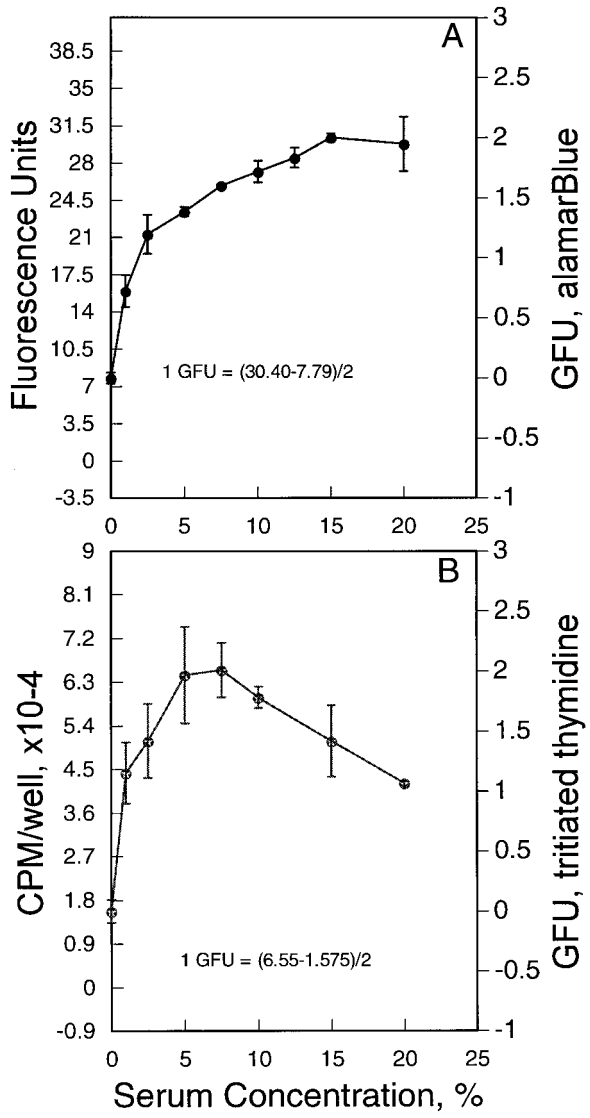

Fig. 1. Comparison of effects of serum on 3T3 fibroblasts in two growth factor assays. Neonatal calf serum standards were tested in **(A)** alamarBlue assay for whole cell proliferation (●) and **(B)** [^{3}H]-thymidine incorporation assay for DNA synthesis (✪). Raw data units are fluorescence (λ = 590) for alamarBlue assay and cpm/well ($\times 10^{-4}$) for [^{3}H]-thymidine. On the right axis the data are expressed as relative growth factor units (GFU) and the conversion equation is given. Each set of data points is the mean ± S.D. of triplicate samples from a single representative experiment.

and results in values less than the baseline recorded for the unstimulated (quiescent) fibroblasts.

The GF activity of the four extracts also was analyzed in dose response curves in the two bioassays (Fig. 3). The activity of the guanidine extract increased with increasing dose up to 100 ug/ml in the alamarBlue assay. The same extract stimulated an increase in the [^{3}H]-thymidine assay which was maximal at 200 µg/ml. The 4 M guanidine extract was unique among the extracts in its ability to generate positive values in both assays. The urea extract of SIS produced dramatically different results. Activity of the urea extract increased with increasing dose to maximum GFU values between 4 and 5 at 400 ug/ml in the tritiated thymidine assay. In contrast, the same extract gave negative GFU values in the alamarBlue assay. As was observed with FGF-2, the dose response curves in these two assays were nearly mirror images. The cellular response to the $MgCl_2$ and NaCl extracts was similar to the response seen with the urea extract (Fig. 3). The $MgCl_2$ and NaCl extracts were less stimulatory than the urea extract, yet were active at a ten-fold lower dose range of 1 to 10 µg/ml. Total dry weight yields (4 to 7 mg/g powdered SIS) or total protein yields (2 to 4 mg/g powder) were not significantly different for these four water-soluble extracts (Table I). Protein content of the extracts typically represented 50–70% of the extract dry weight. Extraction periods longer than 24 h and repeat extractions were tested in an attempt to increase yields. However, little or no additional activity was extractable after the initial 24 h extraction and wash (data not shown).

Identification of FGF-2 as an Extractable Component of SIS

The strong similarity between the GF activity and cell morphology induced by proteins extracted from SIS with 2 M urea and the activity and morphology induced by purified FGF-2 suggested that FGF-2 might be a predominant GF component of this extract. This hypothesis was tested by incubating the extract with increasing amounts of a neutralizing polyclonal antibody specific for FGF-2. A dose-dependent neutralization of GF activity of the urea extract indicated that FGF-2 was present and accounted for more than 60% of the GF activity of this extract as measured in the [^{3}H]-thymidine incorporation assay (Fig. 4).

Neutralization of the activity of the urea extract of SIS with antibody to FGF-2 was apparent also in the changes in morphology of the fibroblast cells (Fig. 5). Swiss 3T3 fibroblasts were cultured in the presence of the extract or purchased GF which had been incubated either

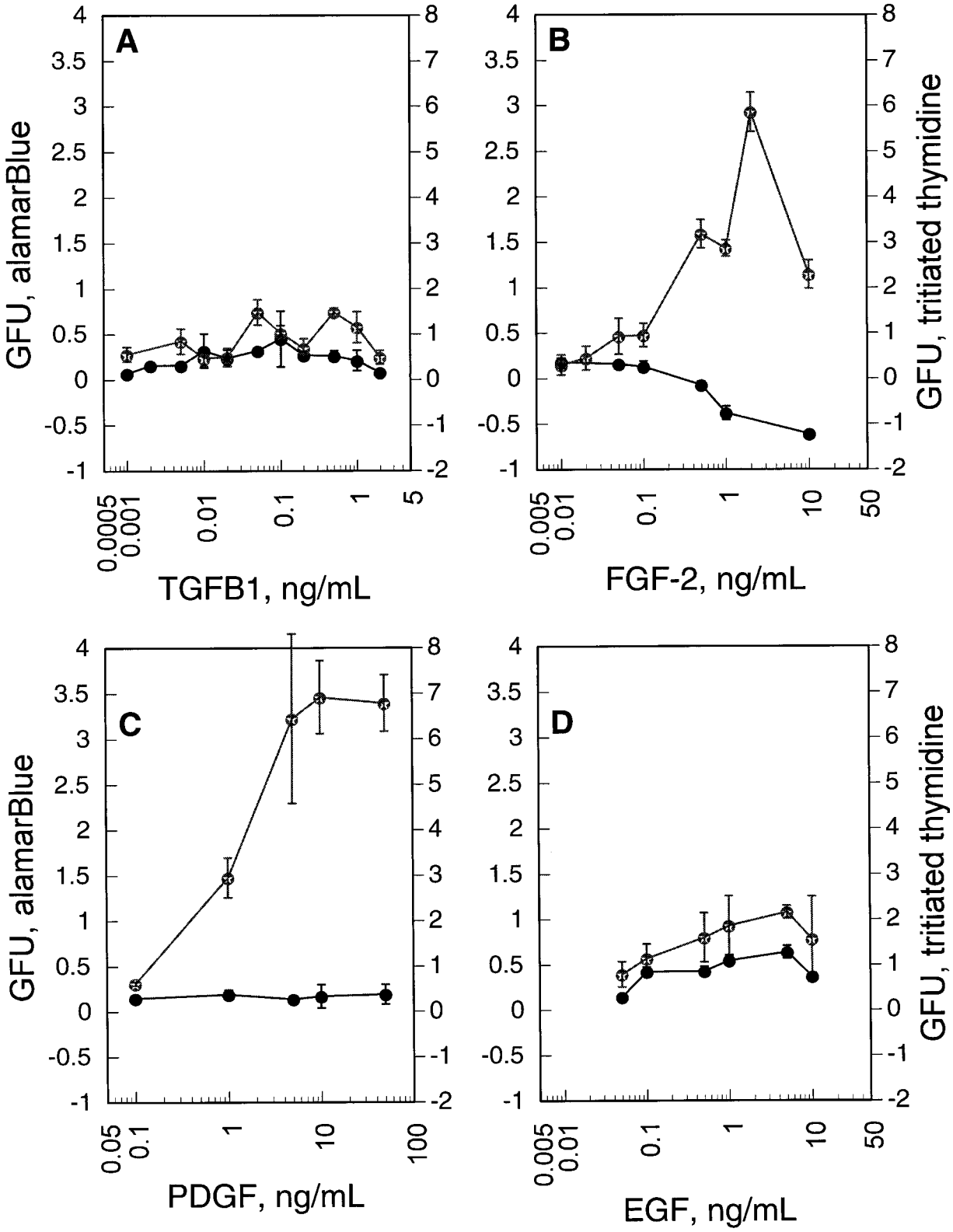

Fig. 2. Responses to purified growth factors in the bioassays. The responses of 3T3 fibroblasts to several purified GFs were determined by measuring whole cell proliferation (alamarBlue assay, ●, left axis) and DNA synthesis ([^{3}H]-thymidine assay, ✪, right axis). Commercially available porcine TGFβ1 **(A),** bovine FGF-2 **(B),** porcine PDGF **(C),** and human EGF **(D)** were tested over a range of concentrations known to be effective with fibroblasts.

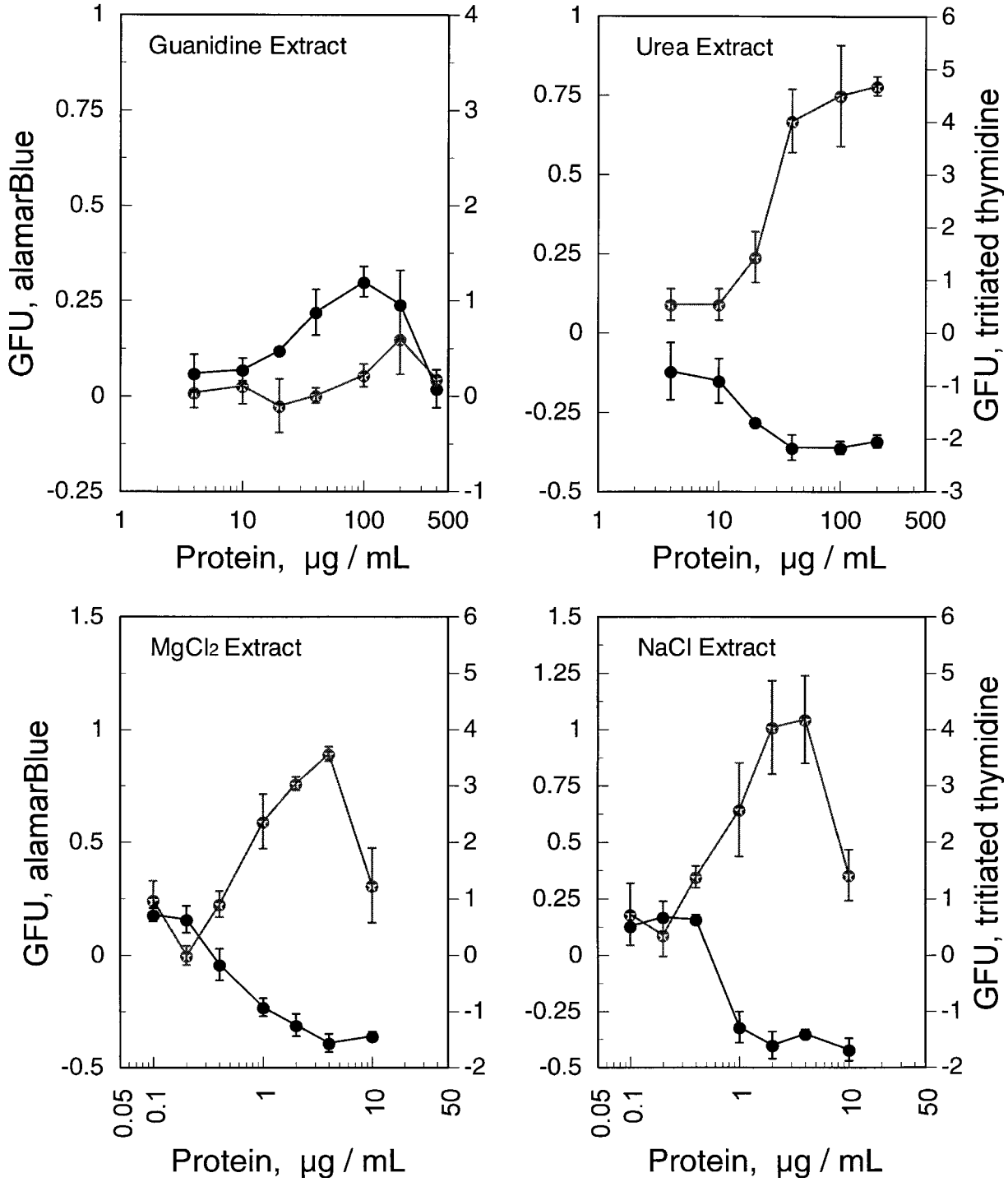

Fig. 3. Comparison of the effects of SIS extracts on fibroblasts in the two growth factor assays. Dose-response curves for extracts of SIS in alamarBlue assay (●) and [^{3}H]-thymidine assay (✪) indicate the differences in range and activity of the various extracts. The same preparations of extracts were used in both assays.

with neutralizing antibody or PBS alone. Compared to either 20% serum (Fig. 5A) or the no serum (Fig. 5B) control, the response of the fibroblasts to purified FGF-2 was quite distinctive showing an increased nuclear to cytoplasmic ratio and a more rounded appearance (Fig. 5C). The cells incubated with urea extract had an appearance similar to those incubated with purified FGF-2 (Fig. 5E). Neutralization of FGF-2 with anti-FGF-2 blocked the change in

TABLE I. Total Yields of Extracts of Porcine SIS

Extraction	Total dry weight	Total protein
	mg/g powder	*mg/g powder*
4 M Guanidine	5.1 ± 0.5	3.1 ± 0.95
2 M Urea	3.7 ± 2.2	2.4 ± 1.2
2 M $MgCl_2$	6.9 ± 3.0	3.95 ± 0.95
2 M NaCl	7.7 ± 3.7	3.75 ± 0.75

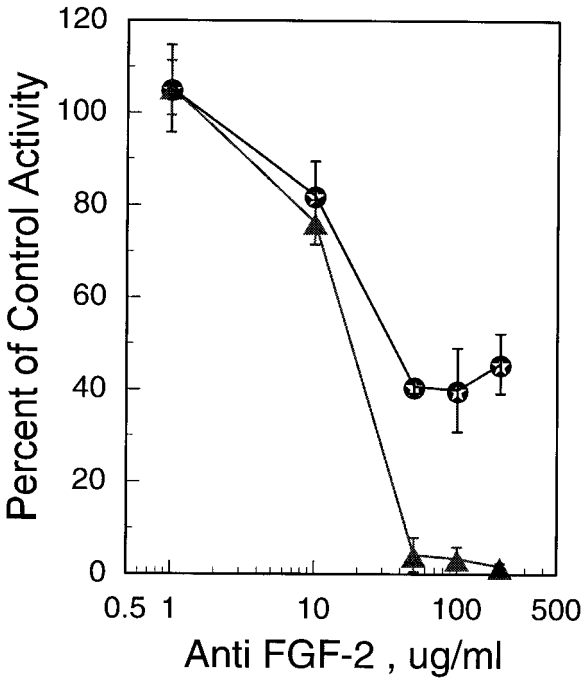

Fig. 4. Neutralization of activity with a growth factor specific antibody. Neutralization of FGF-2 activity of standard FGF-2 (▲) and of 2 M urea extract of SIS (✪) in [^{3}H]-thymidine assay. Data are the average of two experiments. Values for 100% control activities (no antibody) were 3.55 ± 0.03 GFU at 1 ng/ml FGF-2 and 4.96 ± 0.05 GFU at 0.2 mg/ml urea extract.

the cell morphology such that the cells appeared quiescent (Fig. 5D). Incubation of the urea extract with anti-FGF-2 also altered the cell morphology from that normally induced by urea extract (Fig. 5F). The appearance of the fibroblasts was that of a stimulated cell population, but unlike the appearance of the cells with serum.

The presence of FGF-2 in the urea extract of SIS was confirmed further in Western blot analysis (Fig. 6). A monoclonal antibody specific for FGF-2 was used to probe for the protein in the extract after separation on SDS-PAGE. A doublet of reactive bands was reproducibly detected at approximately 19 kDa in the urea extract.

Identification of a TGFβ-Related Protein as an Extractable Component of SIS

The extracts of SIS were screened for TGFβ activity with a neutralizing antibody specific for all the TGFβ isoforms (β1, β1.2, β2, β3, β5). GF activity was neutralized in the extract made with 4 M guanidine. The result of incubating the extract with increasing amounts of antibody was a decreasing activity which eventually reached a minimum in the negative values in the alamarBlue assay (Fig. 7).

The cells incubated with guanidine extract (Fig. 8E) had an appearance very similar to those incubated with the purified TGFβ1 (Fig. 8C), but were more numerous and more extended. The response of the 3T3 fibroblasts to TGFβ1 was more subtle than the response to FGF-2, yet still evident, especially at the low cell densities of the alamarBlue assay. Cells were more flattened and spread-out compared to the serum stimulated (Fig. 8A) or quiescent cells (Fig. 8B). In addition, the TGFβ1 treated cells appeared less well attached to the culture dish than those cultured in the presence of the guanidine extract. Incubation of TGFβ1 with anti-TGFβ prior to addition to quiescent cells prevented the change in morphology of the cells (compare Fig. 8D with C). Incubation of guanidine extract with anti-TGFβ altered the morphology of the cells (Fig. 8F) compare to those treated with guanidine extract alone. However, the cells still appeared to have a different morphology than quiescent cells with many of cells taking on an elongated, spindle-shaped appearance.

An affinity-purified preparation of the same polyclonal antibody, reactive against TGFβ1, β1.2, β2, β3, and β5, was used to probe for TGFβ in the guanidine extract (Fig. 9). No protein band was detected at 25 kDa, a molecular weight corresponding to that of the non-reduced purified TGFβ1, β2, and β3 standards. However, higher molecular weight protein bands of the guanidine extract were reactive with this antibody. These bands were also detected in a partially purified fraction of the guanidine extract. Guanidine extract of demineralized bone powder, a known source of TGFβ, yielded an identifiable 25 kDa protein band along with several higher molecular weight bands. No bands were detected in the controls with secondary antibody only (data not shown).

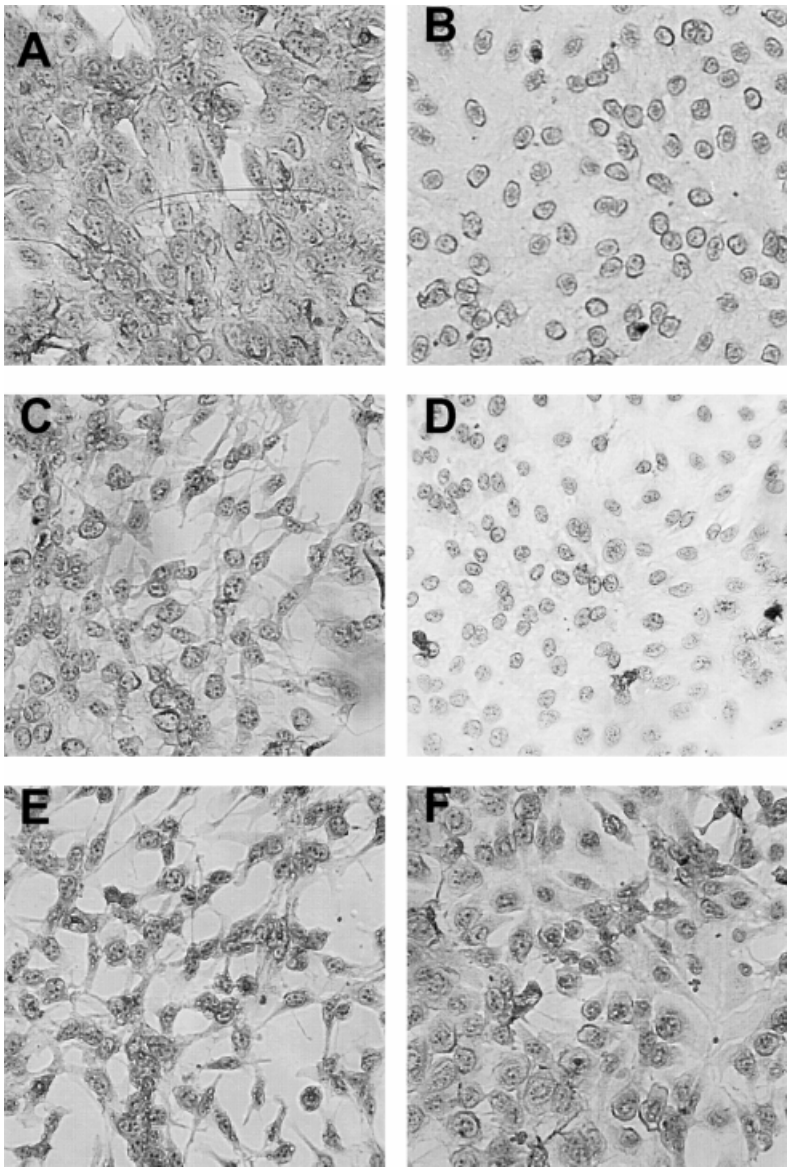

Fig. 5. Changes in fibroblast morphology in response to FGF-2 or urea extract of SIS. Cells were plated and treated as described for [^{3}H]-thymidine assay and then fixed, stained, and photographed. Cells grown in the presence of **(A)** 20% serum or **(B)** 0.1 mg/ml BSA/PBS were used for comparison controls. Cells were treated with, **(C)** FGF-2 at 1 ng/ml, **(D)** FGF-2 incubated with anti-FGF-2 at 0.1 mg/ml, **(E)** urea extract of SIS at 0.2 mg/ml, or **(F)** urea extract incubated with anti-FGF-2 at 0.1 mg/ml.

DISCUSSION

Events of the remodeling response induced by SIS closely approximate those of fetal wound healing and embryogenesis in that appropriate tissue structure and function are restored with minimal scar formation. These inductive properties suggest that SIS is not merely an inert extracellular matrix scaffold but rather that this biomaterial provides "instructive" signals that direct the host cell response. One aspect of this signaling might be related to the presence of growth factors. This present analysis of SIS extracts revealed a significant amount of growth factor activity present in SIS. Growth factors have been identified in gastrointestinal tissues previously. Nice and coworkers [1991] isolated a N-terminally extended form of FGF-2 from the cellular layers of porcine colonic mucosa. Likewise, TGFα and urogastrone EGF were found to be present in human gastrointestinal mucosa, albeit at significantly lesser quantities [Cartlidge and Elder, 1990]. The present study is the first demonstration of growth factors associated with the connective tissue (extracel-

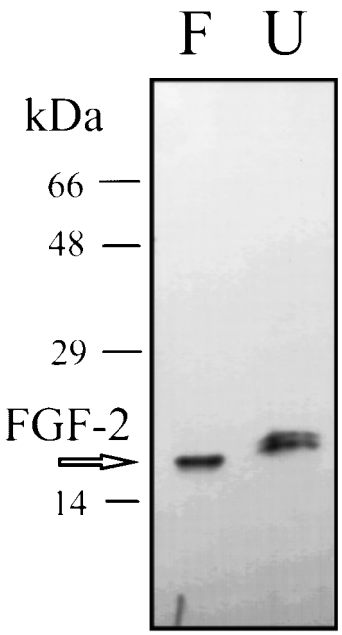

Fig. 6. Immunodetection of FGF-2 in extracted proteins of SIS. Urea-extracted proteins of SIS (U) were separated on 16.5% SDS-PAGE (100 µg/lane) and electro-blotted to PVDF paper. Detection of FGF-2 was with a monoclonal antibody to FGF-2 (1:10,000). Purified human recombinant FGF-2 (25 ng) was used as a standard (F) and is marked with an arrow at its molecular weight of 18 kDa.

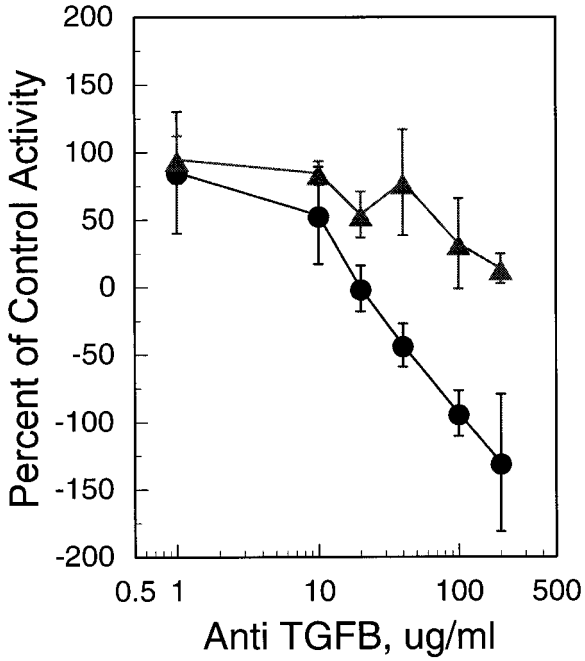

Fig. 7. Neutralization of activity with a growth factor specific antibody. Neutralization of TGFβ activity of standard TGFβ1 (▲) and of 4 M guanidine extract of SIS (●) in alamarBlue assay. Data are the average of replicated experiments (TGFβ1, n = 2; guanidine extract, n = 3). Values for 100% controls (no antibody) were 0.23 ± 0.01 GFU at 20 pg/ml TGFβ1 and 0.15 ± 0.05 GFU at 40 µg/ml guanidine extract.

lular matrix) portion of the small intestine, more specifically the submucosal layers.

Further characterization of the bioactivity within SIS extracts revealed the presence of FGF-2 and a TGFβ-related component. Although maximal neutralization with anti-FGF-2 demonstrated that this GF was responsible for the majority of the measurable activity of the urea extract, considerable GF activity remained. Currently, we are determining if any other known GFs are responsible for this activity. Likewise, residual activity was observed when the TGFβ-related activity of the guanidine extract was completely neutralized. The negative values observed after TGFβ neutralization (in the alamarBlue assay) suggested the presence of FGF-2 activity which might have been previously masked by the TGFβ-related activity. However, dual neutralization of the guanidine extract with antibodies to both TGFβ and FGF-2 revealed the FGF-2 was not responsible for the residual activity (data not shown). The presence of an inhibitory molecule (e.g., Decorin) which was masked by the TGFβ stimulatory activity in this complex extract appears likely [Yamaguchi et al., 1990].

Western blot analysis confirmed the presence of a protein with a molecular weight in the range consistent with previously identified FGF-2. The slightly larger form of FGF-2 might be a differentially expressed form as seen with porcine [Nice et al., 1991], guinea pig [Moscatelli et al., 1987], and human hepatoma [Prats et al., 1989] tissues. N-terminal sequence analysis is necessary to determine homology of this protein to previously identified forms of FGF-2.

The polyclonal antibody used to detect TGFβ recognized several protein bands in guanidine extract of SIS at molecular weights considerably higher than observed for purified TGFβ standards (25 kDa) or extract of bone. It is not clear which, if any, of these proteins are responsible for the TGFβ-related activity of guanidine extract of SIS. That one of the previously reported isoforms of TGFβ is present in the guanidine extract of SIS, but at a level below detection by the Western blot, is still a possibility. However, initial separation of proteins of the guanidine extract of SIS with gel filtration chromatography, based on a protocol for isolating TGFβ [Yamaguchi et al., 1990], also did not reveal a 25 kD band, but did demonstrate that the antibody-reactive higher mol. wt. proteins stay with the TGFβ neutralizable activity

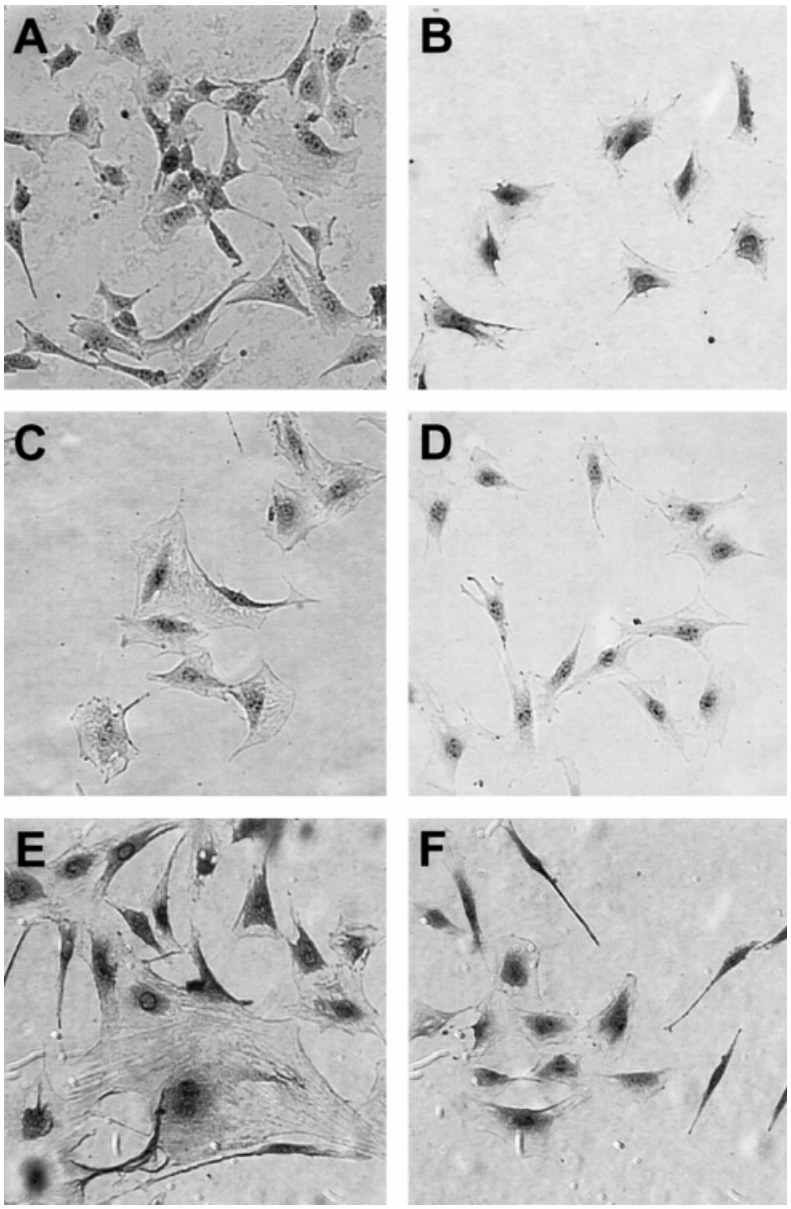

Fig. 8. Changes in fibroblast morphology in response to TGFβ1 or guanidine extract of SIS. Cells were plated and treated as described for alamarBlue assay and then fixed, stained, and photographed. Cells grown in the presence of **(A)** 20% serum or **(B)** 0.1 mg/ml BSA/PBS were used for comparison controls. Cells were treated with, **(C)** TGFβ1 at 20 pg/ml, **(E)** guanidine extract of SIS at 40 μg/ml, **(D)** TGFβ1 incubated with anti-TGFβ at 0.1 mg/ml or **(F)** guanidine extract incubated with anti-TGFβ1 at 0.1 mg/ml.

through one step of purification. Determination of whether SIS contains a very low level of one of the known isoforms of TGFβ, a yet unidentified form of TGFβ or a novel TGFβ-like GF requires further purification and characterization.

It is significant that the two growth factors thus far implicated as being active in extracts of SIS, FGF-2, and a TGFβ-related protein, have been shown to play significant roles in the mechanisms underlying tissue remodeling and wound healing. FGF molecules stimulate the proliferation of most of the major cell types involved in wound healing, including vascular endothelial cells, fibroblasts, smooth muscle cells, osteoblasts and chondrocytes. One of the most striking properties of FGF-2 is its ability to stimulate the proliferation and migration of capillary endothelial cells and all other events associated with angiogenesis. FGF-2 is dependent on binding to heparan sulfate, a gly-

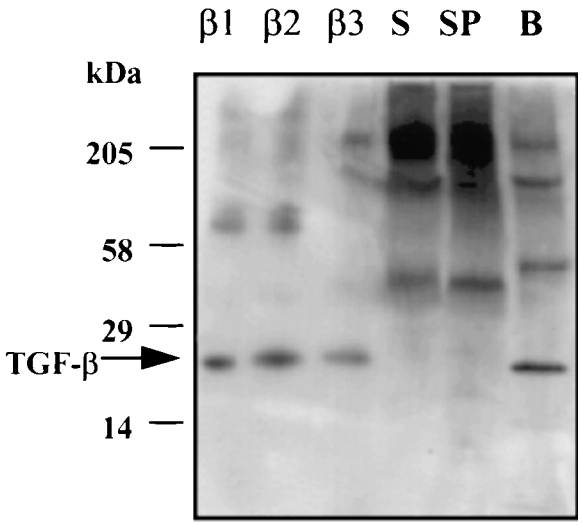

Fig. 9. Immunodetection of TGFβ-related protein in extract of SIS. TGFβ standards (β1–10 ng, β2–20 ng, β3–20 ng), guanidine extracts of bovine bone (B-40 ug) and porcine SIS (S-160 µg), and an active fraction (SP-200 ug), partially purified from guanidine extract of SIS by column chromatography (see Materials and Methods), were separated on 4–20% SDS-PAGE and electroblotted to PVDF paper. Detection of TGFβ was with an affinity purified pan-specific polyclonal antibody to TGFβ (1: 1,000). Secondary antibody coupled to HRP was at 1:15,000.

cosaminoglycan, for high affinity binding to its receptor [Rapraeger et al., 1994; Miao et al., 1995]. Analysis of tissues for FGF-2 often reveals this factor bound to heparan sulfate proteoglycans associated with extracellular matrix and cell surfaces [Aviezer et al., 1994; Brown et al., 1995]. The binding of FGF-2 within ECM might regulate the activity of FGF by acting as a potential storage and release site and by potentiating its effects on target cell receptors [Bashkin et al., 1989; Flaumenhaft et al., 1989; Rapraeger et al., 1994].

On the other hand, TGFβ is perhaps the most potent stimulator of chemotaxis known, stimulating the migration of monocytes, lymphocytes, neutrophils, and fibroblasts in femtomolar concentration range. In addition, the effects of TGFβ on extracellular matrix are more complex and more profound than those of any other growth factor and are central to its effect of increasing the maturation and strength of wounds and of pathologic matrix accumulation characteristic of fibrotic disease [Roberts and Sporn, 1996]. TGFβ regulates the transcription of a wide spectrum of matrix proteins including collagen, fibronectin, and glycoaminoglycans, matrix-degrading proteases, and their inhibitors, yielding a net increase in matrix protein accumulation [Quaglino et al., 1990; Wahl et al., 1993]. TGFβ has been identified previously in ECM of tissues other than intestine. For example, TGFβ has been shown to be stored in bone matrix [Ogawa et al., 1992] through an association with decorin, a proteoglycan component of extracellular matrix [Takeuchi et al., 1994]. Binding to decorin has been shown to regulate the bioactivity of TGFβ [Yamaguchi et al., 1990]. In other studies, TGFβ has been co-localized with decorin to the ECM of various developing tissues [Lysiak et al., 1995]. Recently, a latent form of TGFβ was found associated with latent TGFβ binding protein in the ECM of fibroblast and bone cell cultures [Taipale et al., 1994; Dallas et al., 1995].

What role do these specific growth factors play in the inductive properties of SIS? SIS induces a rapid cellular infiltrate within hours of its implantation and stimulates an early neovascularization. However, it is uncertain as to the contribution of the SIS-derived GFs versus those GFs derived from host cells and tissue. We are presently conducting studies to answer this question.

Preliminary biochemical analysis [Hodde et al., 1996] indicates that the composition of SIS is similar to that of other extracellular matrix structures, which consist of a complex array of collagens, proteoglycans, glycosaminoglycans, and glycoproteins. Therefore, unlike most materials used for tissue engineering, this biomaterial more closely approximates the complexity of natural tissue composition and architecture and thereby provides a scaffold conducive to necessary molecular and cell signaling. An initial understanding of the composition of active factors present in this biomaterial is critical to the investigation of the molecular signals being exchanged between the implant material and the host tissue. As well, the identification of FGF-2 and a TGFβ-related component in extracts of SIS is a significant step toward understanding the mechanisms behind the novel properties of SIS as a wound healing agent and tissue regenerative biomaterial.

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