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Heat shock induces the release of fibroblast growth factor 1 from NIH 3T3 cells

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ABSTRACT **Fibroblast growth factor 1 (FGF-1) is a potent angiogenic and neurotrophic factor whose structure lacks a classical signal sequence for secretion. Although the initiation of these biological activities involves the interaction between FGF-1 and cell surface receptors, the mechanism responsible for the regulation of FGF-1 secretion is unknown. We report that murine NIH 3T3 cells transfected with a synthetic gene encoding FGF-1 secrete FGF-1 into their conditioned medium in response to heat shock. The form of FGF-1 released by NIH 3T3 cells in response to increased temperature (42°C, 2 hr) *in vitro* is not biologically active and does not associate with either heparin or the extracellular NIH 3T3 monolayer matrix. However, it was possible to derive biologically active FGF-1 from the conditioned medium of heat-shocked NIH 3T3 cell transfectants by ammonium sulfate fractionation. The form of FGF-1 exposed by ammonium sulfate fractionation is similar in size to cytosolic FGF-1 and can bind and be eluted from immobilized heparin similarly to the recombinant human FGF-1 polypeptide. Further, the release of FGF-1 by NIH 3T3 cell transfectants in response to heat shock is reduced significantly by both actinomycin D and cycloheximide. These data indicate that increased temperature may upregulate the expression of a factor responsible for the secretion of FGF-1 as a biologically inactive complex that requires an activation step to exhibit the biological activity of the extracellular polypeptide mitogen.**

The fibroblast growth factor (FGF) family of heparin-binding proteins is composed of two prototype members, FGF-1 (acidic) and FGF-2 (basic), and five related proteins (1). The FGF prototype structures are unique among the members of the FGF family because, unlike the majority of FGF-related polypeptides, the FGF prototypes lack a classical signal peptide sequence for secretion (2, 3). The structure of the FGF-1 mRNA and its translation product is the least complicated among FGF family members because not only does it lack a signal sequence but the FGF-1 open reading frame is flanked by termination codons (2). This feature is not found within the structure of the FGF-2 mRNA and, in contrast to the FGF-1 mRNA sequence, the FGF-2 mRNA contains multiple alternative upstream CUG start sites for translation (4–6). Indeed, a similar translational feature is also present in the FGF-3 mRNA (7). Because FGF-1 is a potent inducer of neovascularization *in vivo* (8) and the biological activity of FGF-1 is mediated by an interaction with high-affinity cell surface receptors (1), the mechanism utilized by FGF-1 to gain access to the extracellular compartment may provide insight into the regulation of FGF-1 biological activity *in vivo*.

The heat shock protein (HSP) family of genes encode multifunctional polypeptides whose importance is emphasized by their ubiquitous phylogenic presence, conserved structures, and tightly regulated activation mechanism (reviewed in ref. 9). Indeed, members of the HSP70 family have many functions (9), including the ability to associate with polypeptides known to be directed to specific cellular organelles such as the nucleus (10), nucleolus (11), mitochondria (12, 13, 14), microsomes (15), endoplasmic reticulum (13), and lysosomes (16). Further, the HSP70 and HSP90 family members have been demonstrated to be associated with cellular and viral regulatory polypeptides (reviewed in refs. 9, 17, and 18), including c-Myc (19), p53 (20), adenovirus E1A (20), the glucocorticoid receptor complex (21), clathrin triskelions (22), and polyoma middle T antigen (23, 24). While the expression and translocation of HSP70 between organelles is cell cycle-specific (25) and has been reported to be associated with physiologic vascular stress (26–28), the function of these stress proteins in diseases associated with fever, inflammation, cellular hypertrophy, or programmed cell death (29–32) remains unknown. Because (*i*) the expression of FGF-1 is exaggerated in inflamed cartilage *in vivo* (33), (*ii*) FGF-1 is a potent regulator of cellular hypertrophy (34), and (*iii*) programmed cell death has been proposed as a mechanism for the release of cytosolic FGF-1 (1–3, 8), we examined the role of heat-induced stress as a potential mechanism for the secretion of FGF-1 *in vitro* and report here that the release of cytosolic FGF-1 is regulated by temperature *in vitro*.

MATERIALS AND METHODS

Cell Culture. Murine NIH 3T3 cells were obtained from the American Type Culture Collection, transfected with the eukaryotic expression vector pMEXneo (35), containing the coding sequence for amino acids 21–154 of FGF-1 as described (36), and grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO) containing 10% (vol/vol) fetal bovine serum (HyClone) and G418 (400 μg/ml; GIBCO). Murine BALB/c 3T3 cells and human umbilical vein endothelial cells (HUVECs) were also maintained and grown as previously described (37). For those studies where the growth-promoting activity of NIH 3T3 cell-derived conditioned medium was assessed with HUVECs, the NIH 3T3 cells were maintained and grown in medium 199 (GIBCO) containing 10% fetal bovine serum and heparin (5 units/ml; Upjohn).

Heat Treatment and Processing of Conditioned Medium. NIH 3T3 cell monolayers transfected with the pMEXneo vector with and without the FGF-1-(21–154) insert were maintained under the cell culture conditions described above. Prior to heat shock, the medium was changed to DMEM (serum-free) and the monolayer was incubated at 42°C for 2 hr. The medium was collected and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma) was added. Spectrophotomet-

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Abbreviations: FGF, fibroblast growth factor; HSP, heat shock protein; HUVEC, human umbilical vein endothelial cell.
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ric enzymatic analysis (475 mm) of lactate dehydrogenase (Sigma) in the NIH 3T3 cell control and heat-shocked conditioned media served as an indicator of cell lysis. Conditioned media were collected, centrifuged at 4000 × *g* for 5 min, subjected to 0.22-μm filtration, and processed over a 2-ml heparin-Sepharose 4B (Pharmacia LKB) column equilibrated in 50 mM Tris·HCl, pH 7.0/10 mM EDTA (TEB). The column was washed with TEB, and bound material was eluted with TEB containing 1.5 M NaCl. The eluate was dialyzed against TEB and concentrated (Centricon 10; Amicon) to a volume of 40 μl. In some experiments, the conditioned medium was fractioned with 90% (of saturation) ammonium sulfate (Sigma), as described (38), prior to heparin-Sepharose affinity chromatography. The ammonium sulfate pellet was collected by centrifugation (9000 × *g*, 40 min), suspended in TEB, and dialyzed against TEB for 18 hr at 4°C.

Cell Growth and DNA Synthesis Assays. The mitogenic activity present in heat-shocked NIH 3T3 cell conditioned medium was assessed by a BALB/c 3T3 cell DNA synthesis assay (36) and a HUVEC growth assay (37). Briefly, BALB/c 3T3 cells were grown to confluence and maintained for 48 hr in DMEM containing 0.5% fetal bovine serum. Conditioned medium from control or heat-shocked NIH 3T3 cell pMEXneo:FGF-1 transfectants was added at various concentrations, and the cells were incubated at 37°C for 18 hr. Then [*methyl*-^{3}H]thymidine (0.5 μCi/ml; New England Nuclear; 6.7 Ci/mmol; 1 Ci = 37 GBq) was added and the cells were incubated a further 6 hr. The cells were washed with 0.1 M sodium phosphate buffer, pH 7.0/150 mM NaCl and the trichloroacetic acid-precipitable radioactivity was quantitated as described (36). In the HUVEC growth assay, cells were plated on fibronectin-coated (5 μg/cm^2) cell culture dishes at 10^4 cells per cm^2 as described (37). Briefly, HUVECs were supplemented with 10% fetal bovine serum and heparin (5 units/ml) with or without NIH 3T3 cell conditioned medium, the cultures were fed every 2–3 days, and the viable cells were counted after 12 days (36). HUVECs grown in the presence of human recombinant FGF-1-(21–154) at 10 ng/ml (36, 38) served as a positive control.

Immunoblot Analysis of NIH 3T3 Cell Conditioned Media. Conditioned media derived from control and heat-shocked NIH 3T3 cells were processed as described above. Proteins were resolved by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE; 15% acrylamide) and transferred to nitrocellulose filters as described (36). The filters were incubated with 10 mM Tris·HCl, pH 8.0/150 mM NaCl, 5.0% (wt/vol) bovine serum albumin/0.1% (vol/vol) Tween 20 for 2 hr at 42°C, washed three times with 10 mM Tris·HCl, pH 8.0/150 mM NaCl/0.1% Tween 20 (TCB) and incubated with rabbit anti-human FGF-1 antibody (33) at 1 μg/ml for 18 hr at 4°C. The filters were washed three times with TCB and the proteins recognized by the antibody were detected with an ^{125}I-protein A detection system (Amersham). Human recombinant FGF-1-(21–154) was prepared as described (36, 38) and served as positive control.

RESULTS

NIH 3T3 cells transfected with the pMEXneo:FGF-1 vector (39) were used to study the secretion of FGF-1. No FGF-1 was detected in the medium conditioned by control populations of NIH 3T3 cells transfected with the pMEXneo vector without insert (data not shown) or with the vector containing the FGF-1 insert, when the medium was analyzed by immunoblot analysis after adsorption to and elution from heparin-Sepharose (Fig. 1A, lane 1). Likewise, immunoblot analysis of the heparin-Sepharose eluate demonstrated the absence of FGF-1 in the conditioned medium of heat-shocked (42°C for 2 hr) NIH 3T3 cell pMEXneo:FGF-1 transfectants (Fig. 1*A*, lane 2). Direct immunoblot analysis or immunoprecipitation

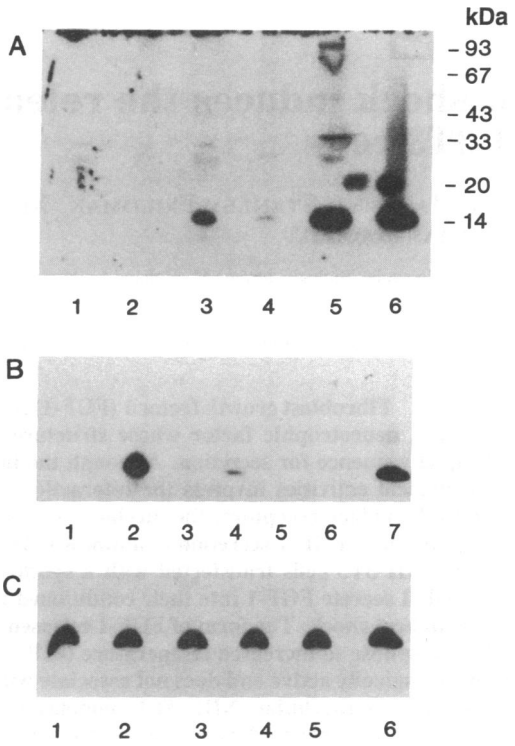

FIG. 1. Immunoblot analysis of FGF-1 from media conditioned by heat shock. (*A*) NIH 3T3 cell pMEXneo:FGF-1 transfectants were grown to confluence. Serum-free DMEM was added at time zero and the monolayer was maintained at 42°C for 2 hr. Control NIH 3T3 cell pMEXneo:FGF-1 transfectants were maintained under identical conditions at 37°C. The conditioned media were collected, 1 mM phenylmethylsulfonyl fluoride was added, the media were applied to heparin-Sepharose, and the eluates were analyzed by immunoblot analysis for FGF-1. Lane 1, conditioned medium from 37°C control cells; lane 2, conditioned medium from 42°C heat-shocked cells; lane 3, conditioned medium from 42°C heat-shocked cells extracted with ammonium sulfate prior to heparin-Sepharose adsorption; lane 4, conditioned medium from 37°C control cells extracted with ammonium sulfate prior to heparin-Sepharose adsorption; lane 5, FGF-1 content of cytosol derived from 37°C control cells; lane 6, recombinant human FGF-1 (200 ng). (*B*) NIH 3T3 cell pMEXneo:FGF-1 transfectants were grown to confluence. Prior to heat shock, the transfectants were incubated at 37°C for 2 hr with either actinomycin D (10 μg/ml) or cycloheximide (10 μg/ml) and then maintained at either 37°C or 42°C for 2 hr. The conditioned media was collected, extracted with ammonium sulfate, and applied to heparin-Sepharose, and the eluates were analyzed by immunoblot analysis for FGF-1. Lane 1, conditioned medium from 37°C control cells; lane 2, conditioned medium from 42°C heat-shocked cells; lane 3, conditioned medium from 37°C control cells treated with actinomycin D; lane 4, conditioned medium from 42°C heat-shocked cells treated with actinomycin D; lane 5, conditioned medium from 37°C control cells treated with cycloheximide; lane 6, conditioned medium from 42°C heat-shocked cells treated with cycloheximide; lane 7, recombinant human FGF-1 (200 ng). (*C*) NIH 3T3 cell pMEXneo:FGF-1 transfectants were maintained and treated with either cycloheximide or actinomycin D as described in *B*. The cytosolic content of FGF-1 in the monolayer population was determined from whole cell lysates. Lane 1, cell lysate from 37°C control cells; lane 2, cell lysate from 42°C heat-shocked cells; lane 3, cell lysate from actinomycin D-treated cells maintained at 37°C; lane 4, cell lysate from actinomycin D-treated cells maintained at 42°C; lane 5, cell lysate from cycloheximide-treated cells maintained at 37°C; lane 6, cell lysate from cycloheximide-treated cells maintained at 42°C.

of conditioned medium from [^{35}S]cysteine/methionine-labeled heat-shocked pMEXneo:FGF-1 transfectants also failed to demonstrate the presence of extracellular FGF-1 (data not shown). In addition, the conditioned media derived from control and heat-shocked NIH 3T3 cell pMEXneo:

FGF-1 transfectants stimulated comparable levels of DNA synthesis in BALB/c 3T3 cells (Fig. 2*A*). Further, it is unlikely that the media conditioned by the control and heat-shocked pMEXneo:FGF-1 transfectants contained an inhibitor of FGF-1 mitogenic activity, since the addition of exogenous recombinant FGF-1 to these conditioned media supported the growth of HUVECs *in vitro* (Fig. 2*D*). These data suggest that the total active growth factor content of the conditioned medium was not significantly affected by heat shock.

Because it was possible that extracellular FGF-1 may have associated with a structure that blocked its ability to remain soluble and recognize immobilized heparin, monolayers of control and heat-shocked NIH 3T3 pMEXneo:FGF-1 transfectants were washed with medium containing heparin at 5 units/ml. Immunoblot analysis of the heparin-washed monolayers failed to detect the presence of FGF-1 as an extracellular matrix-associated polypeptide prior to or after heat shock (data not shown). Further, we were unable to detect increased levels of growth factor activity in the conditioned medium following heat shock and heparin treatment (Fig. 2*B*). However, the biological activity detected by the BALB/c 3T3 cell assay in medium conditioned by either control or heat-shocked NIH 3T3 pMEXneo:FGF-1 transfectants was not FGF-like, since neither of these conditioned media was able to promote HUVEC proliferation *in vitro* (Fig. 2*C*). We also examined the ability of a 2 M NaCl wash to dissociate FGF-1 from the pMEXneo:FGF-1 transfectant monolayer, and under these conditions immunoblot analysis did not show FGF-1 associated with the extracellular compartment (data not shown). Further, the levels of the endogenous FGF-1 mRNA (data not shown) and the intracellular FGF-1 polypeptide (Fig. 1*C*, lanes 1 and 2) were not altered in the pMEXneo:FGF-1 transfectants exposed to heat shock, and these cells remained viable following heat shock. Indeed, analysis of lactate dehydrogenase activity in the conditioned medium before and after heat shock of the pMEXneo:FGF-1 transfectants suggested that cell death did not contribute to the release of cytosolic-associated FGF-1, because (*i*) there was no difference between the relatively low levels of extracellular lactate dehydrogenase activity that were observed in media conditioned by either control or heat-shocked pMEXneo:FGF-1 transfectants (data not shown) and (*ii*) we did not observe FGF-1 biological activity or FGF-1 protein following heparin-Sepharose adsorption (Fig. 1*A*, lanes 1 and 2; Fig. 2 *A* and *B*) in the conditioned medium derived from heat-shocked pMEXneo:FGF-1 transfectants.

To examine the possibility that FGF-1 was complexed to a soluble factor which inhibited the biological activity of FGF-1 and prevented its association with heparin-Sepharose, we used extraction conditions previously successful for the extraction of FGF-1 from bovine tissue (38). Thus, media conditioned by control and heat-shocked NIH 3T3 pMEXneo:FGF-1 transfectants were subjected to ammonium sulfate fractionation (90%, wt/vol) followed by heparin adsorption and immunoblot analysis. While we did not observe a significant level of FGF-1 in the medium conditioned by the pMEXneo:FGF-1 transfectants that were maintained at 37°C (Fig. 1*A*, lane 4; Fig. 1*B*, lane 1), a significant level of extracellular FGF-1 was readily detected in the medium conditioned by the pMEXneo:FGF-1 transfectants that were stressed with heat (Fig. 1*A*, lane 3; Fig. 1*B*, lane 2). Comparative immunoblot analysis using recombinant human FGF-1 as a standard suggested that in the medium conditioned by heat shock, ≈1 ng of FGF-1 per ml was present that was released from cellular stores that approximated 30 ng/ml in the lysate of the pMEXneo:FGF-1 transfectants. In addition, the appearance of FGF-1 in medium conditioned by the pMEXneo:FGF-1 transfectants following heat shock could be effectively reduced by actinomycin D (Fig. 1*B*, lanes 3 and 4) or cycloheximide (lanes 5 and 6). Our failure to detect significant levels of extracellular FGF-1 in media conditioned

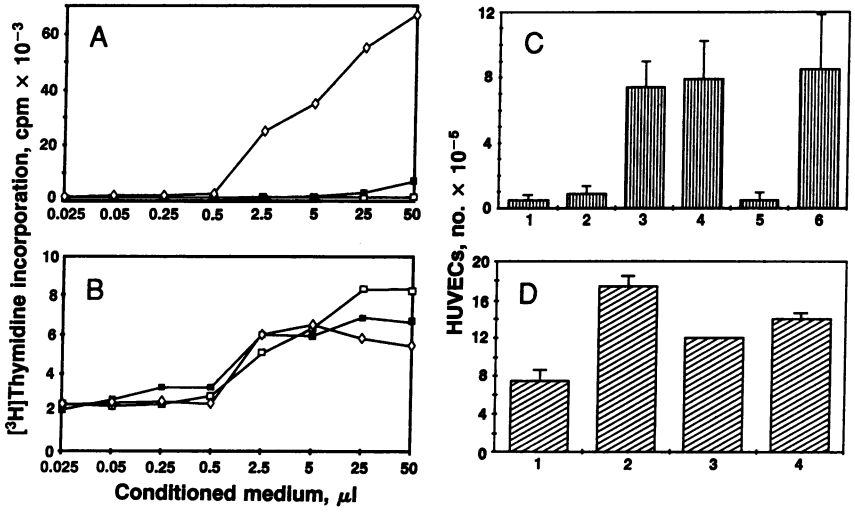

FIG. 2. Biological activity of media conditioned after heat shock. (*A*) Incorporation of [³H]thymidine into DNA by BALB/c 3T3 cells as a function of the concentration of conditioned medium. Conditioned media were derived from NIH 3T3 pMEXneo:FGF-1 transfectants maintained at 37°C (□) or 42°C (■) for 2 hr. DMEM containing 10% fetal bovine serum (◇) served as a cell-free control. (*B*) DNA synthesis assay performed as described in *A*, using conditioned medium from NIH 3T3 pMEXneo:FGF-1 transfectants maintained at 37°C (◇) or 42°C (■) for 2 hr, except that heparin (5 units/ml) was added to the conditioned media and the media were concentrated ≈100-fold. DMEM containing 10% fetal bovine serum (□) served as a cell-free control. (*C*) HUVEC proliferation assay, with data reported as viable cell number after 10 days. Bars: 1, conditioned medium from NIH 3T3 cell pMEXneo:FGF-1 transfectants maintained at 37°C for 2 hr; 2, conditioned medium from cells maintained at 42°C; 3, conditioned medium from cells maintained at 42°C that was extracted with ammonium sulfate; 4, conditioned medium from cells maintained at 42°C that was supplemented with recombinant human FGF-1 (20 ng/ml); 5, 10% fetal bovine serum control; 6, 10% fetal bovine serum control with recombinant human FGF-1 (20 ng/ml). (*D*) HUVEC proliferation assay performed as described in *C*. Bars: 1, conditioned medium from NIH 3T3 pMEXneo transfectants maintained at 37°C for 2 hr that was supplemented with recombinant human FGF-1 (20 ng/ml); 2, same as bar 1, but with the addition of heparin (5 units/ml); 3, conditioned medium from NIH 3T3 pMEXneo transfectants maintained at 42°C for 2 hr that was supplemented with recombinant human FGF-1 (20 ng/ml); 4, same as bar 3, but with the addition of heparin (5 units/ml).

by the pMEXneo:FGF-1 transfectants treated with either cycloheximide or actinomycin D further supports our suggestion that neither cell injury nor cell death contributes to the appearance of extracellular FGF-1 in this system (Fig. 1*B*, lanes 3 and 5). Further, the form of FGF-1 extracted by ammonium sulfate was able to stimulate HUVEC proliferation (Fig. 2*C*) and possessed a molecular weight similar to that of the cytosolic form of FGF-1 (Fig. 1*A*, lanes 3 and 5; Fig. 1*C*).

DISCUSSION

The mechanism used by FGF-1 to exit the intracellular compartment has been an area of interest because FGF-1 lacks a classical signal sequence for secretion (2). We have previously shown by immunohistochemical methods that FGF-1 protein expression is exaggerated *in vivo* in tissues sensitive to inflammation and angiogenesis (33). Those studies were particularly informative because little extracellular FGF-1 protein was observed *in vivo* and the intracellular locale of FGF-1 protein appeared to be both cytosolic and nuclear (33). While these data led to the identification of a FGF-1 nuclear translocation sequence (37, 39), further studies using NIH 3T3 cell FGF-1 transfectants *in vitro* have suggested that cytosolic FGF-1 does not enter the nucleus in a direct manner; rather, nuclear translocation requires the ability of FGF-1 to associate with its high-affinity receptor at the cell surface (39). Thus, these results suggested that the ability of FGF-1 to associate with the nucleus *in vivo* required that FGF-1 be secreted, and because inflammation *in vivo* is usually accompanied by fever (29), we examined the ability of FGF-1 to exit its intracellular compartment in response to increased temperature.

Our data indicate that FGF-1 expressed *in vitro* remains associated with the cytosol (39) and is released into the extracellular compartment in response to heat shock. The secreted form of soluble extracellular FGF-1 is not active as either a BALB/c 3T3 cell or a HUVEC mitogen, nor is it able to bind immobilized heparin. Thus, it is unlikely that the secreted form of FGF-1 is able to associate with its receptor or low-affinity binding sites present either at the cell surface or within the extracellular matrix. Because the process of heat-induced FGF-1 release from the cytosol can be inhibited by both actinomycin D and cycloheximide without influencing cytosolic levels, it is reasonable to suggest that temperature upregulates the expression of a FGF-1-binding protein or influences posttranslational regulatory factors involved in mediating the formation of a FGF-1 complex that directs the secretion of the signal sequence-less polypeptide mitogen. While we do not know the biochemical character of the factor responsible for binding to FGF-1, it is likely that the association between this factor and FGF-1 involves a high-affinity interaction, since neither heparin nor the FGF-1 receptor is able to dissociate FGF-1 from the factor. However, the ability of ammonium sulfate to release FGF-1 from this complex argues that the association process may not involve the formation of a covalent bond between the factor and FGF-1. Further, the observation that FGF-1 can be released from this complex as a heparin-binding growth factor argues that dissociation of the inactive FGF-1 complex may represent a regulatory pathway for the "activation" of extracellular FGF-1. Although the biological mechanism responsible for the activation of latent extracellular FGF-1 is not known, the involvement of extracellular hydrolytic enzymes or another modification of the biologically inactive FGF-1 complex by a posttranslational mechanism is anticipated. Indeed, it is interesting that (*i*) the release of FGF-2/heparan sulfate complexes involves proteolytic activity mediated by plasminogen activators (40, 41), (*ii*) ATP induces the release of FGF-1 from bovine retinal rod outer segments (42, 43), (*iii*) HSP70 has been characterized as both an ATP- and a heparin-binding protein, and (*iv*) digestion of native HSP70 by trypsin generates a large, 47-kDa fragment (9). However, attempts using immunoprecipitation methods to identify FGF-1 complexed to either HSP70 or HSP90 were not successful. Further, we can offer no reason for our failure to detect FGF-1 antigen in the total concentrated medium conditioned after heat shock other than that it may have been destroyed upon concentration of the medium or lost to the plasticware during the concentration process.

We do not know whether temperature is also able to regulate the secretion of other signal sequence-less growth factors such as the interleukin 1 prototypes (reviewed in ref. 44) and FGF-2. However, the release of interleukin 1β from human monocytes is increased in response to heat shock (45), and the inflammatory prostanoids are known regulators of HSP expression in human cells *in vitro* (46). The latter is particularly interesting because the signal-less interleukin prototypes are known to stimulate the expression of cyclooxygenase, a key regulatory enzyme for the synthesis of prostaglandins (47), and because FGF-1 is a potent inhibitor of cyclooxygenase gene expression in human endothelial cells *in vitro* (43) and of the DNA binding of the heat shock transcription factor (48).

The release of FGF-1 in response to increased temperature may also be relevant to physiologic processes responsible for organ/tissue repair and pathologic situations in which both inflammation and angiogenesis are regulated. Indeed, transient ischemia in the heart (31, 32) and brain (26, 30) induces the expression of HSPs, and hyperthermia is a known regulator of HSP expression in a variety of organs and tissues, including skeletal muscle and the aorta (28). In these situations, it is possible that extracellular FGF-1 may function as an important component of repair and disease processes, presumably as a result of its angiogenic and neurotrophic activities (1, 8). Likewise, the secretion of FGF-2 has been implicated in the development of fibrosarcoma in transgenic mice carrying the bovine papillomavirus type 1 genome, and this event correlates with the stimulation of tumor angiogenesis (49). Further, heat shock is able to induce tyrosine phosphorylation *in vitro* (50). Whether other forms of stress, including hypoxia (51), fluid shear (52, 53), or mechanical injury (54), are able to signal the secretion of FGF-1 through the association with a temperature-induced factor remains to be determined.

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