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EXPRESSION OF CONSTITUTIVE AND INDUCIBLE HSP70 AND HSP47 IS ENHANCED IN CELLS PERSISTENTLY SPREAD ON OPN[1] OR COLLAGEN

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Cells persistently spread on OPN or collagen survive heat shock better than cells transiently spread on fibronectin or tissue culture plates. Thus, a central question is whether constitutively or inducible stress proteins are enhanced in cells grown on adhesive proteins that maintain a persistent spread cell shape. Levels of Hsp 72,73, and colligin/Hsp47 were determined by Western blot analyses. The inducible Hsp 72 was prominently expressed following heat shock in cells grown on OPN or collagen, but not in cells plated on fibronectin coated substratum or on tissue culture plates. Colligin/Hsp 47 and Hsp 73 manifested a similar pattern of expression indicating that these adhesive attachment proteins accommodate cell function through organization of cell architecture.

Cell adhesion to a supporting substrate is important to many physiological and pathological events. Encompassed among these are wound healing (1), embryogenesis (2,3), and malignant invasion and metastasis (4-6). To achieve these cellular functions between the cell surface and the substratum, receptor complexes exist that recognize specific matrix proteins (7-9). After contact with a matrix component, cells initially attach and then, in time, reorganize their cytoskeletons to assume a flattened spread shape. A family of receptors has been identified, the integrins, that bind to RGD sequences present in adhesive proteins. The interaction of these receptors with ligands is frequently required for cell attachment and early stages of cell spreading (10). Recently, we have shown that OPN, a secreted phosphoprotein that contains an RGD sequence, promotes both primary cell attachment and later stages of spreading and adhesion (11). This later effect persisted for 96 hours in serumless media after which the cells remained viable. The preservation of a persistent spread morphology was not block by pretreatment of the cells with cycloheximide

[1]OPN has also been termed osteopontin, secreted phosphoprotein (Spp1), 2ar, BSPI and pp69.

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(12). Furthermore, we showed that cells that persistently spread on OPN survive heat shock better than cells demonstrating transient spreading on fibronectin coated surfaces or on tissue culture plates. These findings supported previous studies which have suggested that there exists a link between enhanced tolerance of cells to stress and adhesions proteins (11).

The transcriptional activation of heat shock genes represents a rapid response to environmental and physiological stress and can occur in the absence of protein synthesis (13). Thus, a central question is whether cells persistently spread on an adhesive protein containing a sequence for an integrin receptor can lead to an enhanced expression of constitutively or inducible stress proteins (Hsps). In this study we demonstrate that the inducible and constitutive Hsp70 family of proteins represented by Hsp72 and Hsp73, and colligin/Hsp47, an intracellular collagen binding protein that is a constitutive and heat inducible, are enhanced in cells that are persistently spread on a substratum coated with OPN or collagen. The expression of these Hsps occurs in an environment that does not apparently perturb protein structure or folding. These studies further support idea that gene expression can be controlled either by cell shape or mechanical forces responsible for creating this shape (14).

MATERIALS AND METHODS

Cell Culture:

Gingival fibroblasts were cultured as previously described (15). In essence, healthy gingival tissues were minced and dispersed on glass slides. The slides were inverted in Leighton tubes, and incubated with Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics, glutamine, and 10% fetal bovine serum (FBS). At confluence these cells were trypsinized and centrifuged and aliquots stored in liquid nitrogen. These are designated T_1 for the first passage. For all experiments, cells were used between T_2-T_6 passage.

To determine whether persistent spreading on adhesive proteins containing a RGD sequence regulated the expression of Hsps, total protein and collagen production, bacteriological petri dishes were coated with optimal concentrations of fibronectin [20 ug/ml] (FN) Sigma, human OPN [5 ug/ml] (courtesy of L. Fisher), or Type I collagen [7.5 ug/ml] (15). Precoated dishes and control dishes were then preincubated in 0.4ml DMEM containing 1 mg/ml of bovine serum albumin (BSA) for 1 h at 37°C. Following the preincubation period, cells at a concentration of 1×10^5 in 0.1ml DMEM/BSA were added to the wells. Cells were then maintained in DMEM without serum at 37°C in an atmosphere of 5% $C0_2$. When expression of inducible Hsp was required, the cells were heat shocked at 43°C for 90 minutes and allowed to recover for 4 h prior to harvest (15).

Determination of Total Protein and Collagen:

The methods for labeling fibroblasts have been reported previously (12,15). Briefly, 2×10^4 cells per test well were seeded in DMEM with 10% FBS and antibiotics and the cells incubated overnight. On the following day, medium was removed, and the cells were then incubated for 24 h with fresh medium. Following this incubation, cell layers were rinsed with Hank's balanced salt solution (HBSS) and labeled for 24 h in serum free proline-free DMEM plus 10 uCi/ml L-5-^{3}H-proline. The media and cell samples were then pooled and

extensively dialyzed against cold buffer (pH 7.4, 0.05 mol/liter Tris-HCl, 0.02 mol/liter NaCl, 0.005 mol/liter $CaCl_2$). Following dialysis, aliquots of these samples in Aquasol-2 (New England Nuclear) were counted in a liquid scintillation counter. These resulting radioactive counts represented total nondialyzable radioactive material and were taken as a measure of total protein production.

For estimation of collagen, an aliquot of the above dialyzed material was reacted for six hours at 37°C with chromatographically purified bacterial collagenase (Advanced Biofactures, Form III) according to the methods of Perterkofsky and Diegelmann (16). Undigested proteins were precipitated with the addition of 5% trichloroacetic acid/0.5% tannic acid, and samples centrifuged. Samples were counted in Aquasol-2 (NEN) in a liquid scintillation counter. Background values were subtracted from the collagenase treated series so that a value could be attained representing collagen production expressed as collagenase-digestible protein.

Nonradioactive wells were run in parallel with the above-mentioned radioactive experiments, so that the total number of cells/well at time of harvest could be obtained. These cells were enzymatically removed with 0.08% trypsin/0.04% EDTA, and total cells per well were determined electronically by a Coulter counter. All assays were run in triplicate and statistical significance was determined by ANOVA and Duncan's multiple renge test.

Western Blot Analysis:

For Western blots, the media were removed, the cells were washed with cold phosphate buffered saline, lysed in Laemmli sample buffer, and analyzed by one-dimensional SDS-PAGE (15). The separated proteins were then electrotransferred to nitrocellulose. The paper was blocked with 3% bovine serum albumin in 10 mM Tris-HCL, pH 7.4, 0.9 M NaCl (TBS) for 1 h and then in TBS/BSA with 2% normal goat serum. Antiserum, anti-Hsp47 (courtesy of K. Yamada, NIH/NCI), anti-Hsp72 (inducible form) or anti-Hsp72/73 (constitutive and inducible form) [CBR, Itnl] or preimmune serum were diluted in the same solution and incubated with gentle shaking overnight. The nitrocellulose paper was rinsed 3 times for 5 minutes in TBS/Tween the secondary antibody, affinity purified conjugated with alkaline phosphatase was diluted to 0.9 ug/ml and incubated with the paper for 2 h.

RESULTS

OPN and collagen promoted spreading of cells which was preserved during the 24 h of incubation. In contrast, cells grown on FN maintained spreading 3-6 h subsequent to attachment. At 24 h, cells plated on FN remained attached but were in a rounded pattern which was deviate from the persistent bipolar patterns displayed by cells on OPN and collagen (Figure 1).

To establish whether this persistent attachment could be related to an increase in specific proteins, cells were radioactively labeled and total protein, and collagen production were determined. Total protein production by gingival fibroblasts grown on OPN, collagen, and tissue culture plates is shown in Table 1. Cells grown on OPN and collagen produced minimal but significantly more total protein and more collagen than cells grown on tissue culture plates.

Next, to determine whether adhesive proteins containing a sequence for an integrin receptor enhanced the expression of constitutive and inducible Hsps, Western blot analysis

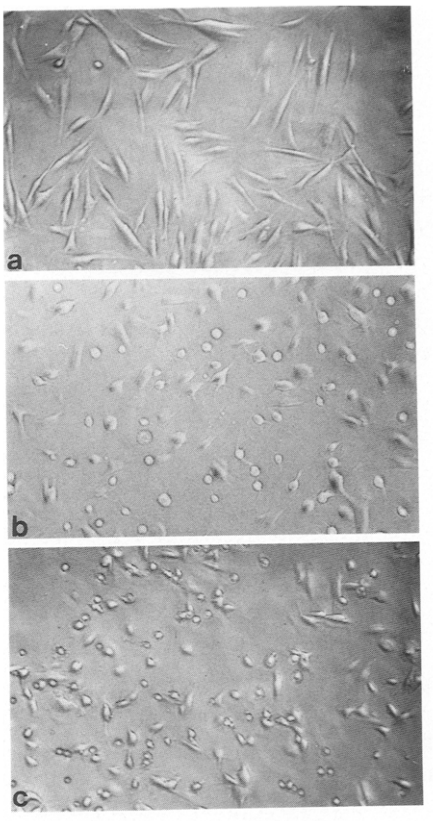

Figure 1. Cells were plated on bacteriologic dishes coated with (a) OPN (5 ug/ml), (b) fibronectin (20 ug/ml) or (c) grown on tissue culture plates as described under "Materials and Methods". Cells grown on OPN reveal persistent bipolar spread shapes while fibronectin treated cells or those on tissue culture plates have either begun to release from the substratum or have kite, rounded or epithelioid shapes. Magnification x15.

was performed on cell lysates analyzed by SDS-PAGE. Figure 2 depicts the total proteins produced by cells grown on collagen, OPN, FN, and control plates separated by SDS-PAGE and stained with Coomassie brilliant blue. These data revealed no differences in the individual protein profiles. However, when the immunoblots of the separations were analyzed by Western blotting utilizing antibodies to Hsp72, Hsp73, and Hsp47 differences were observed between cells persistently spread on OPN and collagen, and cells grown on FN or control plates. The inducible Hsp72 was not expressed in any of the cells despite the substratum on which the cells were propagated. However, when the cells were stress by a heat-shock of 43°C for 90 minutes, bipolar spread morphologies supported by substratum coatings with OPN or collagen revealed prominent staining of Hsp72 (Figure 3). Cells grown on FN were weakly expressive and control cells only exhibited a trace of Hsp72. The constitutive Hsp73 was expressed both in cells persistently spread on OPN and collagen but was only apparent in trace amounts in control and FN treated cells (Figure 3, bottom). This

Table 1

Synthetic Activity of Fibroblasts Grown on OPN and Collagen Coated Plates

Treatment	Protein (cpm x $10^{-3}/10^6$ cells)	Collagen (cpm x $10^{-3}/10^6$ cells)
Control	768 ± 47	17.8 ± 0.7
Collagen (7.5 ug/ml)	821 ± 68*	19.1 ± 0.5*
OPN (5.0 ug/ml)	849 ± 69*	18.2 ± 0.8*

*P < 0.05 significantly different from control cells grown on tissue culture plates. Data expressed as mean ± S.D. and represent the mean of three experiments.

pattern of expression was similar for Hsp47, with cells spread on collagen (Fig. 4a) or OPN (Fig. 4c) showing higher levels of the stress protein than FN or control cells (Fig. 3b,d).

DISCUSSION

In this study we examine whether the preservation of a spread cell shape by native ligands which have been shown to posses a RGD sequences for cell attachment alter the expression of constitutive and inducible Hsps common to fibroblasts. Our work goes on to

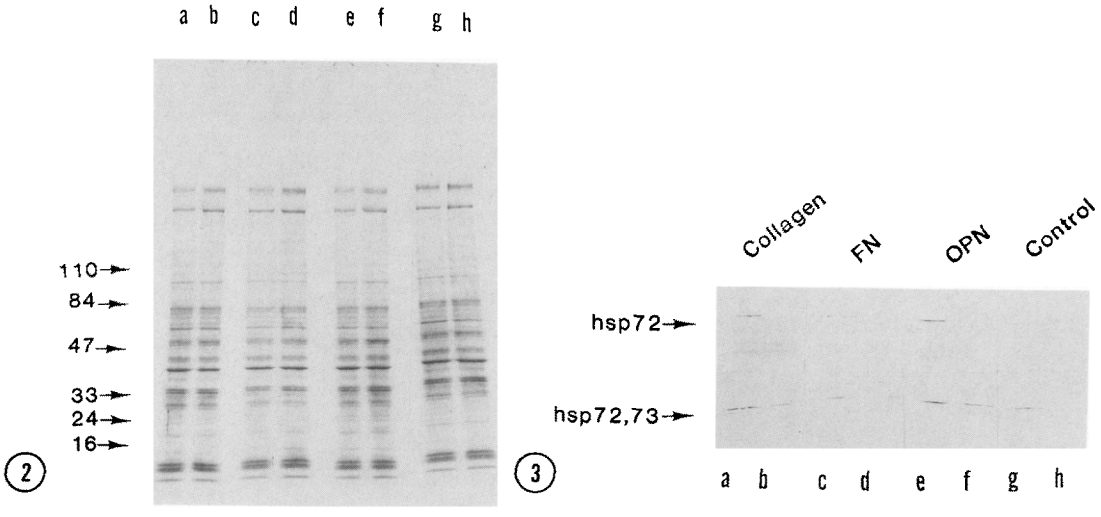

Figure 2. Coomassie brilliant blue stained proteins separated by SDS-PAGE derived from gingival cells grown on various substrata. Collagen coated plates (a) heat treated to express inducible proteins (b) no heat treatment. Fibronectin coated plates (c) heat treated, (d) no heat treatment. OPN coated plates (e) heat treated, (f) no heat treatment, and cells grown on tissue culture plates (g) heat treated and (h) no treatment.

Figure 3. Western blot performed on SDS-PAGE separated proteins derived from gingival cells grown on various coated substrata. Lanes a, c, e, and g represent cells that were induced with 43°C heat while lanes b, d, f, and h were not heat challenged. Western blot analyses are described in "Materials and Methods".

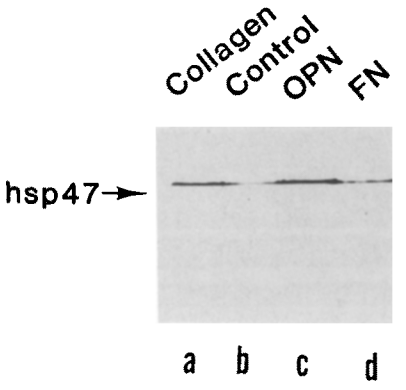

Figure 4. Western blot of SDS-PAGE separated proteins utilizing anti-Hsp47 antibodies. Cells were grown either on bacteriologic plates coated with (a) collagen (7.5 ug/ml), (b) tissue culture plates, (c) OPN (5 ug/ml), or (d) fibronectin (20 ug/ml), as described in "Materials and Methods".

show that both OPN and collagen produce enhanced levels of Hsp72, Hsp73 and Hsp47. This all occurs in spite of only slightly affecting the levels of total protein and collagen and has been previously shown not to be inhibited by cycloheximide (12).

The cause and relationship between persistent spreading and tolerance to heat, though still unclear, may be related to enhanced expression of the Hsps. This idea is support by a number of previous reports which have indicated that Hsps levels are increased in direct proportion to the severity of the stress (17,18), and that preexisting amounts of Hsp72,73 are protective and influence Hsp synthesis following a second stress event (18). However, the mechanism by which Hsps are enhanced by cell shape alterations has yet to be defined. Previous studies indicate most inducers of the stress response require posttranslational modifications that may include phosphorylation, disruption of a factor ligand complex (13), or protein conformational changes (19). The studies reported here that point to a correlation between shape and attachment of cells rather than an alteration of gene products, suggest that Hsp72/73, and Hsp47 gene expression may be related to some posttranslational modification of a pre-existing transcription factor in response to a change in cytoarchitecture (13). The signal for these modifications could include alterations in tension-sensitive ion channels in the plasma membrane that are perturbed by spreading (14). Recently, Mosser et al. (13) have demonstrated that changes in calcium concentration result in concentration- and time-dependent activation of heat shock transcription factors. Also, these conditions reduce the temperature dependence for heat shock factor (HSF) activation. Alternatively, the expressions of Hsps may also be posttranslationally autoregulated following changes in the equilibrium between free and substrate-bound forms of Hsps that occur as a result of a organization of cytoskeletal proteins form monomeric states to polymeric unites (20).

The fact that several attachment proteins containing a common RGD sequences direct alternative spread patterns, i.e. kite shapes, epitheloid and bipolar patterns (21), suggests that these proteins may be accommodating a specific cellular function through organization of their cell shapes. This regulation may be independent from the RGD domain. Fibronectin, and OPN both contain RGD sequences within their primary structure and collagen is especially rich in RGD sequences. Thus, the interaction between integrin receptor and adhesive protein to produce a persistent spread pattern may not be completely mediated through the RGD sequence within these proteins. In fact, there is precedent for such integrin receptor action. GPIIb-IIIa, a major integrin receptor on blood platelets, has been shown to bind peptides composed of the fibrinogen γ chain sequence, lacking a RGD sequence. Moreover, GPIIb-IIIa will also bind fibinogen A α chain residues containing a RGD region, indicating that the same integrin molecule can bind distinct and spatially distinct sites within a single adhesive protein (22). To date, integrin receptors for OPN and collagen have not been completely characterized to determine if receptors associated with persistent spreading operate in a manner analogous to platelet receptors. In spite of this, the formation of prominent bipolar patterns resulting in the persistent anchorage of cells utilizing integrin receptors with a resulting tolerance to stress is seen as an important characteristic for tissue maintainance.

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