Apoptosis and Survival of Osteoblast-like Cells Are Regulated by Surface Attachment

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We tested the hypothesis thatRGDS peptides regulate osteoblast survival in culture. Osteoblast-like MC3T3-E1 cells were allowed to attach toRGDS peptides that had been tethered to a silicone surface utilizing a previously described grafting technique. The RGDS-modified surface caused up-regulation of α5β1 integrin. We noted that there was an increase in expression of activated focal adhesion kinase and activated Akt. There was no change in the expression level of the anti-apoptotic protein Bcl-2, the pro-apoptotic protein Bad, or the inactivated form of Bad, pBad. Attachment to the RGDS-treated membrane completely abolished apoptosis induced by staurosporine, the Ca2+·P3 ion pair, and sodium nitroprusside. However, the surface modification did not interfere with apoptosis mediated by the free RGDS peptide or serum-free medium. When the activity of the phosphatidylinositol 3-kinase pathway was inhibited, RGDS-dependent resistance to apoptosis was eliminated. These results indicated that the binding of cells to RGDS abrogated apoptosis via the mitochondrial pathway and that the suppression of apoptosis was dependent on the activity of phosphatidylinositol 3-kinase.

Osteoblast survival requires attachment to specific extracellular matrix proteins, a phenomenon termed anchorage dependence (1). The survival signal is mediated by transmembrane integrin receptors that provide a mechanical link between the cell and the hydrophilic peptide sequence Arg-Gly-Asp (RGD) on extracellular macromolecules (2, 3). This amino acid motif enhances initial osteoblast attachment and spreading (4, 5). In addition to influencing downstream maturation events (6), RGD signaling (7) induces mineralization of the extracellular matrix (8, 9). Likewise, exposure of osteoblasts to immobilized RGDS peptides increases osteoblast maturation (9–12); at millimolar concentrations, free RGD-containing peptides induce skeletal cell death (13).

The interaction between the RGD motif and integrin receptors causes phosphorylation of the non-receptor protein-tyrosine kinase and activation of focal adhesion kinase (FAK) (14, 15). Once phosphorylated, FAK provides binding sites for Src-homology-2 domains of the survival signaling pathway molecules Grb2-Sos and phosphatidylinositol 3-kinase (PI3K) (16, 17). Of the downstream targets of PI3K, Akt plays a critical role in the regulation of the balance between apoptosis and survival. Once the Akt pathway is activated, there is stimulation of a number of downstream events that include: phosphorylation and activation of caspase-9, resulting in inhibition of apoptosis and enhancement of cell survival (18, 19); maintenance of the mitochondrial membrane potential and suppression of mitochondrial-initiated apoptosis (20); and phosphorylation of Bad and prevention of mitochondrial dysfunction (18, 21). In addition, activated Akt phosphorylates a number of cell survival-related transcription factors, including Forkhead 1 (22), NF-κB (23, 24), and Creb (25).

Despite overwhelming evidence that relates integrin-mediated adhesion with cell survival (26), the relationship between adhesion and the regulation of bone cell apoptosis is poorly understood. In this study, we address this issue by examining the cell line, as our previous studies have shown that these cells exhibit an apoptotic response identical to that of primary osteoblasts (13, 27). Other workers have demonstrated that this cell line mimics the major phenotypic characteristics of osteoblasts (28). Changes in intracellular signaling molecules, FAK, phospho-FAK, Akt, phospho-Akt, Bad, phospho-Bad, and Bcl-2, were examined. Western blot analysis was also used to evaluate changes in expression of Bad and Bcl-2. Rather than evaluate a wide range of integrins, we focused on α5β1 by Western blot analysis. These integrins are known to be expressed by MC3T3-E1 cells as well as other osteoblast-like cells and primary osteoblasts derived from bovine, rat, and human bone samples.

MATERIALS AND METHODS

Study Design

The goal of this investigation was to evaluate the mechanism by which RGDS-mediated integrin attachment promotes the survival of osteoblast-like cells. We chemically bonded anRGDS peptide to a functionalized silicone membrane. A similar RGES-grafted surface was used as a control substrate. MC3T3-E1 cells were then grown on the RGDS and control surfaces. We chose to use this osteoblast-like cell line, as our previous studies have shown that these cells exhibit an apoptotic response identical to that of primary osteoblasts (13, 27).

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Moreover, they are expressed at all stages of development. To evaluate the impact of the peptide on susceptibility to apoptosis, cells were cultured on the RGD-grafted surface for 5 days and then treated by serum starvation or with low doses of the following agents: staurosporine, the Ca²⁺-P i ion pair, sodium nitroprusside or free RGDs peptide. These agents were selected for two reasons: they are effective osteoblast apoptogens, and they activate the extrinsic, as well as the intrinsic, apoptotic pathway. Cell death was determined by the MTT assay. Finally, the importance of PI3K pathway-activated survival signals was assessed by using the specific kinase inhibitor, LY294002.

**Reagents and Antibodies**

Anti-α, and anti-β (polyclonal) integrin antibodies were obtained from Chemicon International Inc. (Temecula, CA). Anti-phospho-FAK (Tyr397) rabbit anti-serum and anti-F-FAK rabbit polyclonal antibodies were provided by Upstate Biotechnology (Lake Placid, NY). Anti-Akt and phospho-Akt to Akt antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-Bad polyclonal, anti-phospho-Bad, and anti-Bcl-2 antibodies were purchased from Sigma-Aldrich. Anti-GAPDH antibody was obtained from Research Diagnostics Inc. (Flanders, NJ). RGDs and RGES peptides were obtained from Sigma. The PI3K inhibitor LY294002, was purchased from Cell Signaling Technology. Protein A-Sepharose beads were from Amersham, and gel blotting material was obtained from Invitrogen.

**Preparation of RGD-treated Silicone Membranes**

RGD peptides were grafted to silicone surfaces utilizing a technique described previously (9) based on a method originally reported by Dee et al. (7, 29, 30). Briefly, 0.005-inch silicone sheets (Silastic Q7-4840, Specialty Manufacturing Inc., Saginaw, MI) were exposed for 10 min to UV light (1000 mW/cm²) using LYO Cleaner, Jeltech Company Inc, Irvine, CA) to functionalize and oxidize the surface. The functionalized silicone surface was then modified by treatment with 0.2 mM 3-aminopropytriethoxysilane (A3648, Sigma) in hexane for 45 min. The 3-aminopropytriethoxysilane molecule reacted with the OH groups, generating an ethoxysilane (A3648, Sigma) in hexane for 45 min. The 3-aminopro-

**Western Blot Analysis**

Western blot analysis was performed as described above for assessing the expression of FAK and phosphorylated FAK (antibody concentration 1:500), Akt and phospho-Akt (antibody concentration 1:500), Bad, phosphorylated Bad, and Bcl-2 (antibody concentration 1:300). GAPDH was used as a loading control.

**Evaluation of Signaling Pathways**

The protocol described above was followed for assessing the expression of FAK and phosphorylated FAK (antibody concentration 1:500), Akt and phospho-Akt (antibody concentration 1:500), Bad, phosphorylated Bad, and Bcl-2 (antibody concentration 1:300). GAPDH was used as a loading control.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

RT-PCR was performed by the GeneAmp RNA PCR kit (PerkinElmer Life Sciences). The PCR products were analyzed by electrophoresis using a 2% agarose gel. Primers for Bad (forward, AGCTAGACACACCATCTCT, and reverse, TTCAAGGTCATGACATGCT, Bcl-2 (forward, CCACTATGGTCACCTGAC, and reverse, ACCCGATCCATCACCC), and a control, GAPDH (forward, GGGAACCCTACAT, and reverse, GCACGCGCCTCACCC) were used.

**Assessment of Apoptotic Sensitivity**

After 3 days in culture on the prepared surfaces, cells were incubated overnight with sublethal doses of apoptogens. Untreated cells and cells plated on the RGDs surface were used as controls. Cell death was measured using the MTT assay (36, 37). This assay is based on the ability of mitochondrial dehydrogenases to oxidize thiazolyl blue (MTT) molecule in the presence of reduced tetrazolium salts. The blue formazan product is proportional to the dehydrogenase activity, a decrease in the absorbance at 590 nm provides a direct measurement of the number of viable cells. To determine the contribution of the PI3K pathway to inhibition of apoptosis, some cell populations were pretreated with 50 µM LY294002, a PI3K inhibitor (38, 39). Following this pretreatment, cell death was determined as described above.

**Statistical Analysis**

Experiments were repeated three to five times. Similar results were seen with each of the replicates. Data were analyzed using a one-way analysis of variance; the Student-Newman-Keuls post hoc test for a comparison of individual means was used. p < 0.05 was considered to be statistically significant.

**RESULTS**

Osteoblast Binding to RGD-grafted Surfaces Up-regulates Integrin Expression—Integrin expression levels were evaluated in MC3T3-E1 osteoblast-like cells cultured on RGD-treated membranes after 0.5, 3, 24, and 72 h. Fig. 1 shows that there was an increase in the α,β3 heterodimer expression on the RGDs surface when compared with the RGES controls. Expression levels were raised after 24 h and maintained for 72 h.

Osteoblast Binding to RGD Peptides Up-regulates Survival Pathways—Integrin signaling events that are triggered by osteoblastic attachment to the RGDs-grafted surface were evaluated by measuring Bcl-2, Bad, and FAK expression. FAK (Fig.
A) and phosphorylated FAK (Fig. 2B) were constitutively expressed by cells adherent to both the RGD- and RGE-grafted surfaces. A modest increase in phosphorylated FAK was evident in cells adherent to the RGD-grafted surface (Fig. 2B).

We investigated downstream signaling events mediated by activation of FAK. Western blot analysis showed that for the first 24 h, there was little difference in levels of Akt expressed by osteoblasts on the two surfaces (Fig. 3). However, at 72 h, there was a considerable increase in Akt protein expression in cells cultured on the RGDS surface. Interestingly, there was little difference in activated phospho-Akt on either surface at 0.5 or 3 h. However, by 24 h, there was a considerable increase in activated Akt in relationship to GAPDH. The increase in phospho-Akt was maintained for 72 h. At this time, the increase in phospho-Akt was directly proportional to the increase in total Akt protein.

To further explore downstream signaling events, we examined the expression of the pro-apoptotic protein Bad (Fig. 4A) and the inactive form of the protein, pBad (Fig. 4B). Both proteins were constitutively expressed by the osteoblast-like cells that were adherent to the RGDS- and RGE-grafted surface. No significant changes in Bad or pBad were observed. mRNA analysis confirmed this observation, as no changes in message levels were seen for Bad at any time point (Fig. 4C). Finally, we examined the Bcl-2 protein (Fig. 5A) and mRNA (Fig. 5B) expression. No difference was observed in either Bcl-2 protein or mRNA levels on the RGDS or RGE surface.

**Osteoblast Binding to RGD-grafted Surface Peptides Promote Osteoblast Survival**

How does integrin-mediated adhesion of osteoblasts to the biomimetic surface influence apoptotic sensitivity? To address this question, cells were grown on the RGDS substrate and treated with 0.1 and 0.5 μM staurosporine; no significant cell killing was observed (Fig. 6A). In contrast, staurosporine killed osteoblasts maintained on the RGE surface in a dose-dependent manner. Fig. 6B demonstrates the effect of sur-
face chemistry on the apoptotic activity of the Ca$^{2+}$-Pi ion pair. Although 3 mM Pi and 2.4 mM Ca$^{2+}$ did not kill osteoblasts grown on the RGDS surface, there was a significant increase in cell death on the RGES surface. A further increase in cell death was apparent when osteoblasts were treated with 3 mM Pi and 2.9 mM Ca$^{2+}$. Again, there was no significant increase in death among osteoblasts maintained on the RGDS surface. The effect of the NO donor sodium nitroprusside on osteoblast apoptosis was similar to that of the Ca$^{2+}$-Pi ion pair and staurosporine. Fig. 6C shows that at concentrations of 0.1 and 0.5 mM, sodium nitroprusside killed 45 and 65% of osteoblasts cultured on the RGES surface, respectively. These concentrations of sodium nitroprusside failed to kill osteoblasts on the RGDS surface. In contrast to these agents, free RGDS (Fig. 6D) and serum-free medium (Fig. 6E) killed osteoblasts on both surfaces. The tetrapeptide RGDS killed osteoblasts that were anchored to both the RGDS- and RGES-grafted surface (Fig. 6D). A similar effect was seen when there was serum withdrawal (Fig. 6E). Thus, in the absence of serum, 45 and 60% of osteoblasts cultured on RGES and RGDS were killed, respectively.

Resistance to Apoptosis by RGD-anchored Osteoblasts Is Mediated by PI3K—To test the hypothesis that resistance to apoptosis was mediated by the PI3K pathway, RGDS-anchored cells were cultured in media supplemented with LY294002. When cells adherent to the RGDS surface were then treated with staurosporine, a profound increase in cell death was observed (Fig. 7A), comparable with the response of cells adherent to the RGES surface. A similar response was seen when the MC3T3-E1 cells were treated with the Ca$^{2+}$-Pi ion pair (Fig. 7B). It was evident that when the PI3K survival pathway was blocked, the protection provided by the RGDS surface was lost, and the cells became susceptible to apoptosis. In contrast, when osteoblast-like cells were attached to RGES and treated with the Ca$^{2+}$-Pi ion pair, their apoptotic sensitivity was not affected by the PI3K inhibitor.

**DISCUSSION**

Results of the investigation indicated that attachment of osteoblasts to an RGDS-grafted surface significantly inhibited the activation of apoptosis. The RGDS-grafted surface caused up-regulation of $\alpha_\beta_3$ integrin receptors as well as raised expression of activated FAK. Furthermore, this study revealed that there was a demonstrable increase in activated Akt followed by guest on July 22, 2018http://www.jbc.org/Downloaded from
not influence the activities of key pro- and anti-apoptotic regulatory proteins concerned with survival of osteoblast-like cells adherent to the RGDS surface.

We used a number of different apoptogens to explore the relationship between cell survival and integrin attachment to tethered RGDS peptides. The apoptogen concentrations that were used in this study were shown previously to induce apoptosis in a number of cell types (13, 27, 32–35). By allowing the osteoblast-like cells to attach to the RGDS-modified surfaces over 72 h, we mitigated the immediate signaling effects of integrin binding and observed how attachment modulates the apoptotic response of the tethered differentiating osteoblasts.

**Fig. 6.** Apoptotic sensitivity of osteoblasts adherent to the RGDS-grafted surface. MC3T3-E1 cells were maintained on the RGDS and RGES surfaces for 72 h. Following incubation with apoptogens for 24 h, cell death was analyzed by the MTT assay. Cells were challenged with the following apoptogens: staurosporine (A), Ca²⁺/Pi ion pair (B), sodium nitroprusside (SNP) (C), free RGDS peptides (D), and serum starvation (E). Each bar represents the mean and standard error of the mean (n = 3). *, p < 0.05 when compared with control (both untreated and RGES surface).
attached to tissue culture plastic, the ion pair promotes a mitochondrial membrane permeability transition and a rapid induction of osteoblast apoptosis (27). To confirm that the attachment peptide blocked apoptosis, we treated the osteoblasts with sodium nitroprusside. We had shown previously that this agent induces death via the mitochondrial pathway, and inhibitors of NO synthase block Ca$^{2+}$-Pi ion pair (27). In concert with the ion pair, sodium nitroprusside failed to promote apoptosis of osteoblast-like cells bound to the RGDS-grafted surface. On the basis of these two studies, it is concluded that the surface maintains cell viability by blocking the mitochondrial membrane permeability transition and thereby preserving mitochondrial function.

Of the other apoptotic study, it was noted that staurosporine failed to kill osteoblasts attached to the RGDS membrane. Although staurosporine is a nonspecific protein kinase inhibitor, it does cause a mitochondrial membrane permeability transition (47), and hence its mode of killing is similar to the ion pair (27) and sodium nitroprusside (41). Conversely, serum starvation or treatment with RGDS peptides promoted death of cells attached to both the RGDS- and RGES-grafted surfaces. Although the death pathway activated by free RGDS peptides is not known, it is likely that the peptides directly engage pro-caspase-3, and there is minimal mitochondrial involvement (46). The effects of serum starvation are more complex and probably encompass changes in both mitochondrial function and mitogenic signals (13). This dual effect would explain why some cells responded to this type of apoptogenic challenge.

To test the hypothesis that inhibition of apoptosis was dependent on the activity of PI3K, we treated adherent osteoblast-like cells with the inhibitor LY294002 and then challenged the cells with a number of agents discussed above. We noted that in the presence of the inhibitor, osteoblasts were no longer resistant to the Ca$^{2+}$-Pi ion pair, and more than 60% of the cells were killed. Likewise, when treated with staurosporine and sodium nitroprusside, the tethered cells underwent apoptosis. The results of all of these studies using multiple apoptogens strongly indicate that when osteoblasts are attached to RGDS-tethered surfaces, there is inhibition of apoptotic responses transduced by mitochondria and enhanced survival activated by the PI3K pathway.

Finally, it is important to acknowledge that the extracellular matrix is a very complex, heterogeneous, and dynamic structure, and the number of receptors and receptor subtypes in bone cells is enormous. Nevertheless, the use of a single tethered peptide provides a unique approach to delineating which pathways are activated and permits dissection of the sequence of events that characterize outside-inside signal transduction. Because many of the attachment motifs are now known and linkage chemistries are available, it should be possible to evaluate the contribution of each of the proteins of the extracellular matrix to osteoblast differentiation, maturation, and function. The use of such a system should enhance elucidation of crosstalk between ligands and growth factors and provide access to downstream effector pathways.

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FIG. 7. Effect of the PI3K inhibitor LY294002 on osteoblast survival on the RGDS-grafted surfaces. MC3T3-E1 cells were incubated on the RGDS and RGES surfaces for 72 h in the presence and absence of the PI3K inhibitor LY294002. Following incubation, the cells were stained and the number of live cells was determined.

A. MC3T3-E1 cells were incubated on the RGDS and RGES surfaces for 72 h in the presence and absence of the PI3K inhibitor LY294002. Following incubation, the cells were killed. Likewise, when treated with staurosporine (STS) (A) and the Ca$^{2+}$-Pi ion pair (B) for an additional 24 h. Cell death was measured by the MTT assay. Each bar represents the mean and standard error of the mean (n = 3). *, p < 0.05 when compared with control (both untreated and RGES surface). n.s., not significant.
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