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Rapid Communication

Inhibition of Angiogenesis *In Vitro* by Arg-Gly-Asp–Containing Synthetic Peptide

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This study was designed to evaluate the effect of the synthetic peptide Gly-Arg-Gly-Asp-Ser (GRGDS) on angiogenesis in serum-free collagen gel culture of rat aorta. The GRGDS peptide contains the amino acid sequence Arg-Gly-Asp (RGD), which has been implicated as a recognition site in interactions between extracellular matrix (ECM) molecules and cell membrane receptors. RGD-containing synthetic peptides are known to inhibit attachment of endothelial cells to substrates, but their effect on angiogenesis has not been fully characterized. Aortic explants embedded in collagen gel in the absence of GRGDS generated branching microvessels through a process of endothelial migration and proliferation. Addition of GRGDS to the culture medium caused a marked inhibition of angiogenesis. In contrast, GRGES, a control peptide lacking the RGD sequence, failed to inhibit angiogenesis. The inhibitory effect of GRGDS was nontoxic and reversible. The angiogenic activity of aortic explants previously inhibited with GRGDS could be restored by incubating the cultures in GRGDS-free medium. These findings suggest that angiogenesis is an anchorage-dependent process that can be inhibited by interfering with the attachment of endothelial cells to the ECM. It also indicates that synthetic peptides can be used as probes to study the mechanisms by which the ECM regulates angiogenesis. (Am J Pathol 1991, 138:829–833)

Developing microvessels during angiogenesis produce a heterogeneous extracellular matrix (ECM) composed of different collagen types, glycoproteins, and proteoglycans.[1–3] The molecules of the ECM have profound effects on the attachment, migration, proliferation, and organizational behavior of isolated endothelial cells.[4–7] The endothelial response to the ECM is, in part, mediated by integrins, a family of cell membrane receptors that bind to ECM molecules. Many receptors belonging to the integrin family recognize polypeptide domains containing the Arg-Gly-Asp (RGD) amino acid sequence.[8] The RGD sequence is present in fibronectin, laminin, collagen, thrombospondin, and fibrinogen, all of which can accumulate in the microvascular matrix during physiologic or pathologic angiogenesis.[2,9,10] Synthetic peptides containing the RGD sequence inhibit attachment and migration of endothelial cells *in vitro* by competing with matrix molecules for their integrin receptors.[11–13] Because endothelial attachment and migration are essential components of microvascular development, we hypothesized that the RGD sequence regulates the interactions between sprouting endothelial cells and the surrounding ECM during angiogenesis. We tested this hypothesis in serum-free collagen gel culture using the aortic ring model of angiogenesis.[14] Here we present data demonstrating that Gly-Arg-Gly-Asp-Ser (GRGDS), a synthetic peptide containing the RGD sequence, inhibits angiogenesis. Conversely GRGES, a control peptide containing a glutamic acid residue in place of aspartic acid, has no effect. These findings support the hypothesis that angiogenesis is an RGD-dependent phenomenon.

Materials and Methods

The aortic ring serum-free assay of angiogenesis *in vitro* has been described in detail in a recent paper.[14] Briefly, rings of rat aorta were embedded in gels of rat tail collagen and cultured in serum-free Molecular Cellular and Developmental Biology (MCDB) medium 131, a growth medium optimized for microvascular endothelial cells.[15] The collagen gel cultures were first prepared in cylindrical agarose wells and kept in triplicates at 35.5°C in

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100-mm culture dishes each containing 30 ml of medium. For the assay, on the third day of culture, each collagen gel culture of rat aorta was freed of the surrounding agarose support with a sterile razor blade and transferred to a 16-mm plastic well (four-well Nunc multidish, Interlab, Thousand Oaks, CA) where it was allowed to float freely in 0.5 ml of medium. Purified synthetic peptides purchased from Peninsula Laboratories (Belmont, CA) were reconstituted in serum-free MCDB 131 medium and added to floating collagen gel cultures. The purity of the peptides was indicated by chromatographic characterization on high-pressure liquid chromatography, which showed one peak for each peptide. The effect of the RGD sequence on angiogenesis was studied using the GRGDS peptide. The GRGES peptide, which contains a glutamic acid residue in place of aspartic acid, was used as control. The cultures were kept at 35.5°C and received fresh medium with peptides every other day. Control cultures without peptides also were studied. Angiogenesis was quantitated by counting the newly formed microvessels according to published criteria.[14] Curves of microvascular growth were obtained by recording daily the number of microvessels in each culture.

Results

Angiogenesis in the Absence of Synthetic Peptides

New microvessels first were seen sprouting in the collagen gel from the resected ends of the aortic explants on day 3 of culture. During the next 4 to 5 days the microvessels increased in number and length forming branches and some loops (Figures 1A and 2). Previously we demonstrated that microvessels generated by aortic explants *in vitro* are positive for factor VIII-related antigen,[14] synthesize DNA, and are mitotically active.[16] The newly formed microvessels ceased proliferating on day 7 or 8 and underwent spontaneous regression during the second week (Figure 2).

Inhibition of Angiogenesis by GRGDS Peptide

Treatment of cultures with 300 μg/ml GRGDS in the medium caused a marked inhibition of angiogenesis. Cultures treated with GRGDS were characterized by an abortive growth phase that lasted only 1 to 2 days and resulted in the formation of a significantly reduced number of microvessels that underwent early regression (Figures 1B and 2). The concentration of GRGDS in the medium was critical because angiogenesis was inhibited consistently with 300 μg/ml of the peptide but no effect was seen with 3 μg/ml, 30 μg/ml, or 150 μg/ml.

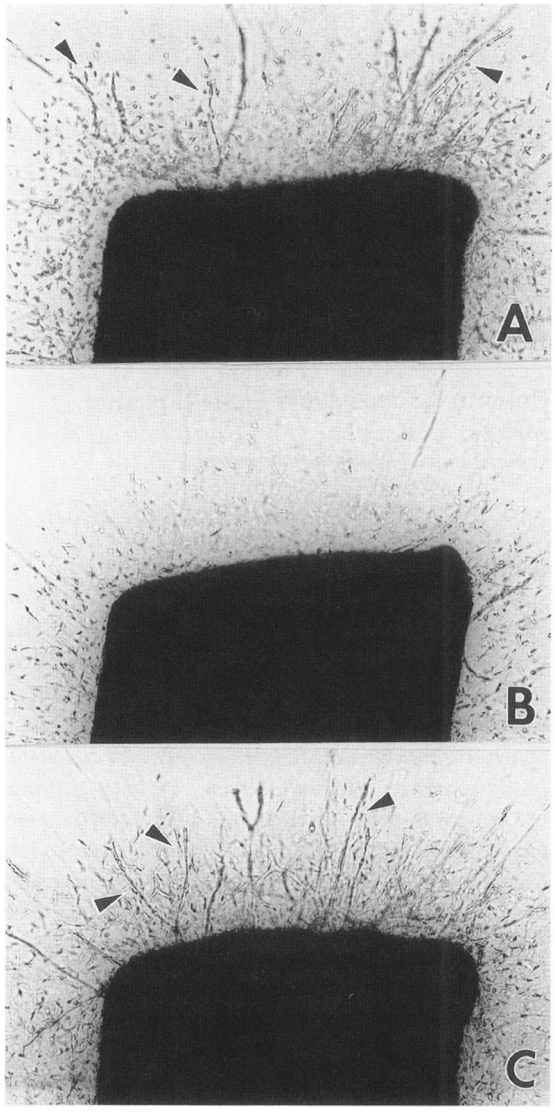

Figure 1. *Effect of RGD-containing peptide on angiogenesis in 7-day-old collagen gel cultures of rat aorta. Angiogenesis is markedly inhibited in the culture treated with 300 μg/ml GRGDS* **(B)**. *In contrast, there are many newly formed microvessels (arrowheads) in the absence of GRGDS* **(A)** *and in the presence of 300 μg/ml GRGES* **(C)** *(×35).*

Specificity of GRGDS Effect on Angiogenesis

To evaluate whether the antiangiogenic effect of GRGDS was RGD dependent, control cultures were treated with 300 μg/ml GRGES, which lacks the RGD sequence. Cultures receiving GRGES behaved as untreated controls, confirming the specificity of the GRGDS effect (Figures 1C and 2).

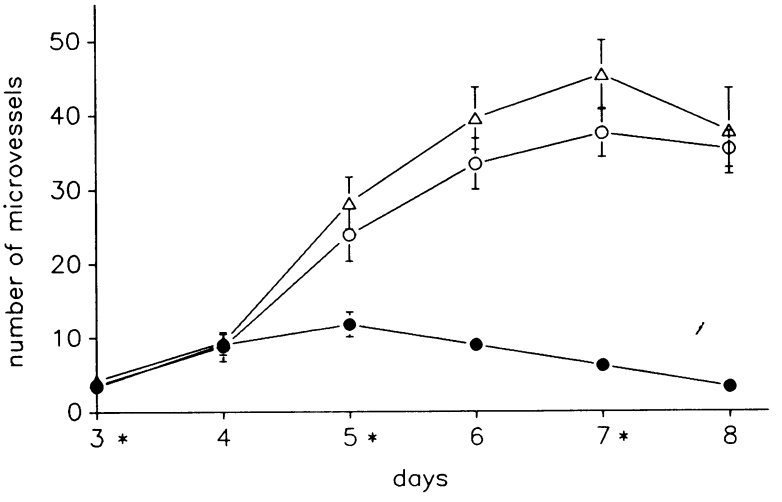

Figure 2. *Effect of GRGDS on microvascular growth curve in collagen gel culture of rat aorta. GRGDS, 300 μg/ml (closed circles); GRGES, 300 μg/ml (open triangles); control without peptides (open circles). Asterisks indicate days of treatment with peptides. Note: There is a significant inhibition of angiogenesis by GRGDS that allows only a limited and abortive growth of microvessels (P < 0.01 at day 7 by ANOVA). Cultures treated with the control peptide GRGES behave as untreated control. Error bars indicate standard error of the mean (n = 9).*

Reversibility of GRGDS-induced Inhibition of Angiogenesis

To maintain the antiangiogenic effect, it was necessary to add fresh GRGDS peptide to the culture every other day when the medium was changed. If the cultures were not treated continuously with the peptide, the aortic rings recovered from the inhibitory effect of GRGDS, producing a delayed angiogenic response. The number of microvessels generated by aortic explants recovered from GRGDS treatment was similar to that of untreated control cultures (Figure 3). This indicated that the GRGDS effect was reversible and nontoxic.

Discussion

This study demonstrates that GRGDS, a synthetic peptide containing the RGD amino acid sequence, inhibits angiogenesis in serum-free collagen gel culture of rat aorta. The RGD sequence is a widespread recognition system used by endothelial and other cells to attach to the ECM through specialized cell membrane receptors called integrins.[17] Previous studies have shown that RGD-containing peptides cause rounding and detachment of endothelial cells from plastic or matrix-coated surfaces by competing with the binding of RGD-containing ECM molecules to cell membrane receptors.[11] Endothelial cells detached with RGD-containing peptides are viable and can be subcultured if the inhibitory peptide is removed from the medium. These observations are consistent with our findings that aortic explants inhibited with GRGDS maintain intact their vasoformative potential and generate microvessels if returned to a GRGDS-free medium.

Endothelial cells during vascular proliferation interact with RGD-containing molecules such as fibronectin, laminin, and collagen.[18] The RGD sequence also is found in von Willebrand factor, thrombospondin, and fibrin, which all may accumulate in the ECM during wound healing and tumor angiogenesis.[19,20] Adhesive interactions between endothelial cells and ECM molecules are believed

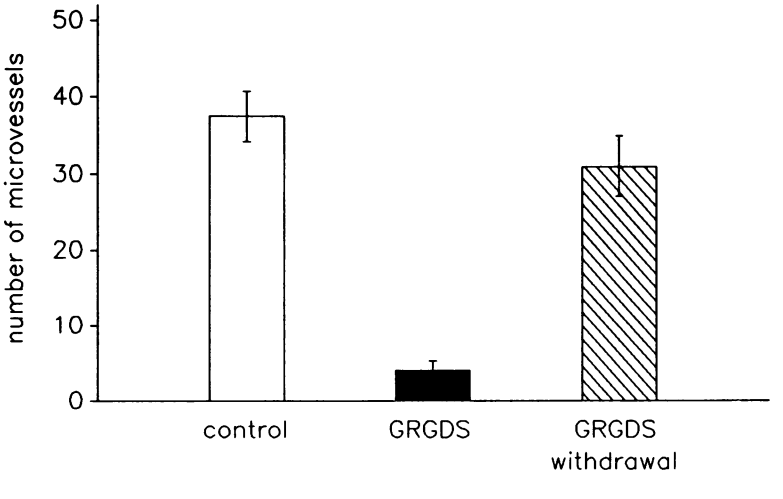

Figure 3. *Reversibility of antiangiogenic effect of GRGDS. Number of microvessels formed in* **(A)** *collagen gel cultures of rat aorta never treated with GRGDS (open bar);* **(B)** *collagen gel cultures of rat aorta treated with GRGDS (closed bar);* **(C)** *collagen gel cultures of rat aorta treated with GRGDS to obtain maximum inhibition of angiogenesis and then allowed to recover in GRGDS-free medium (hatched bar). Note: The inhibitory effect of GRGDS on angiogenesis is reversible. GRGDS-treated cultures have significantly lower number of microvessels as compared to the GRGDS-free and the GRGDS withdrawal groups (P < 0.01 by ANOVA). Error bars indicate standard error of the mean (n = 6).*

to regulate through mechanochemical mechanisms the growth, differentiation, and morphogenesis of microvessels during angiogenesis.[21] Our results support this idea and suggest that angiogenesis is an anchorage-dependent process that can be inhibited by interfering with the RGD-mediated attachment of endothelial cells to the ECM. In a recent study, Grant et al[22] reported that an RGD-containing synthetic peptide of laminin inhibited the reorganization of pre-existing endothelial cells into cords and networks induced by a reconstituted basement membrane substrate. Here we demonstrate that an RGD-containing peptide inhibits angiogenesis in a model in which microvessels form as a result of endothelial migration and proliferation rather than from reorganization of pre-existing endothelial cells. This observation suggests that 1) attachment of endothelial cells to the ECM is an essential early step in angiogenesis; and 2) inhibition of endothelial attachment to the ECM results in the failure of these cells to migrate, proliferate, and form capillary tubes. Reversible inhibition of cell proliferation by an RGD-containing peptide has recently been observed also by Choy et al[23] in three-dimensional aggregate culture of embryonic heart mesenchyme. In addition, RGD-containing peptides have been shown to inhibit amphibian gastrulation and avian neural crest migration in intact embryos.[24]

The relatively high concentration of GRGDS required to inhibit angiogenesis probably is due to the low affinity of the integrin receptors.[13,25] Concentrations of peptides similar to the ones required in our study have been used by others to inhibit the attachment of cells to substrates,[11,13,26] to suppress neural crest migration *in vitro,*[24] and to elute integrin receptors from matrix affinity chromatography columns.[27]

In summary, this study provides support for the hypothesis that angiogenesis is an anchorage-dependent process and demonstrates that GRGDS, a synthetic peptide containing the RGD amino acid sequence, has antiangiogenic activity *in vitro*. It also indicates that serum-free collagen gel culture of rat aorta can be used as an assay to study the mechanisms by which the ECM regulates angiogenesis.

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