The platelet integrin glycoprotein (GP) IIb/IIIa, which mediates platelet aggregation, has been the target for novel antiplatelet agents, the GPIIb/IIIa antagonists. Several GPIIb/IIIa antagonists have been developed based on the peptide RGDS present in adhesion proteins, including the principle ligand fibrinogen. The apoptosis enzyme, procaspase-3, contains an RGD-recognition sequence and is activated by RGDS. We examined the effects of RGDS and several GPIIb/IIIa antagonists on cell death and procaspase-3 activation in rat neonatal cardiomyocytes. These antagonists do not recognize rat integrins, yet RGDS, orbofiban, and xemilofiban induced dose-dependent apoptosis and procaspase-3 activation in cardiomyocytes over 72 h, particularly under hypoxic conditions. Scrambled peptide, the monoclonal antibody 7E3 or integrin (a peptide containing a RGD sequence), had little or no effect. Immunoprecipitation of procaspase-3 followed by treatment with the compounds showed that procaspase-3 was activated directly by RGDS, orbofiban, xemilofiban, and by monoclonal 7E3 antibody, the latter demonstrating that compounds must enter cells to induce apoptosis through caspase activation. Integrin had no effect. Binding studies with 2-SC52012B, a GPIIb/IIIa antagonist analogue of orbofiban, showed no specific binding to cardiomyocytes, but the radioligand accumulated intracellularly over 72 h. 2-SC52012B also bound directly to human recombinant caspase-3 (Kd 5 9 ± 2 nm), and this was prevented by orbofiban, xemilofiban, and the monoclonal 7E3 antibody but not by integrin. Finally, confocal microscopy showed that RGDS co-localized with caspase-3 inside the cell. These data show that RGDS and its mimetics induce cardiomyocyte apoptosis by direct activation of procaspase-3.

Integrins are cell surface molecules that are involved in cell adhesion to matrix proteins and other cells. Integrins are also capable of generating intracellular signals upon engaging their ligands, including signals involved in cell growth and survival (1, 2). Glycoprotein (GP)1 IIb/IIIa is largely confined to platelets and megakaryocytes, although it is also found on some melanoma cells (3). GPIIb/IIIa is required for normal platelet aggregation, an early event in the development of arterial thrombosis (4). Several adhesion proteins bind to GPIIb/IIIa, but the principle ligand is fibrinogen (5). The interaction between fibrinogen and GPIIb/IIIa involves several regions of the ligand, including the motif RGD, two copies of which are found on each α-chain of fibrinogen (6). Several compounds have been designed, based on the RGD sequence, as antagonists of fibrinogen binding (7, 8). GPIIb/IIIa antagonists prevent platelet aggregation and have been found to prevent coronary artery thrombosis, at least when given as short term infusions.

Several members of the procaspase family of apoptosis genes, including procaspases-1, -2, -3, -7, and -8 also contain RGD motifs (9). Two of these, procaspase-1 and -3, also contain potential RGD-binding motifs near to the experimentally determined processing sites necessary for activation to the mature caspase. Indeed, RGD-containing peptides are capable of inducing apoptosis in T-cells through direct procaspase-3 activation without any requirement for integrin engagement or modification of integrin-mediated cell signaling (9). Potentially, RGD-containing peptides act by inducing conformational changes in the procaspase leading to increased oligomerization, a putative step in the activation of the enzyme (10). Similar activation of procaspase-3 in cardiomyocytes may explain the reported increase in cardiac death in patients on the oral GPIIb/IIIa antagonist, orbofiban. Moreover, such a mechanism may explain the proapoptotic properties of RGD peptides in models of angiogenesis, inflammation, and cancer metastasis (11–13).

Activation of caspase-3 is a common ultimate signal in response to factors that induce apoptosis, such as Fas-induced apoptosis in several cell types (14). Caspase-3 also plays an important role in the apoptosis of cardiomyocytes rendered ischemic (15). Caspase-3 activates a number of targets, including nuclear enzymes involved in DNA degradation (16). We investigated if RGD peptides and RGD mimetics induced apoptosis through direct procaspase-3 activation in rat cardiomyocytes. The compounds studied were xemilofiban and orbifiban, designed to mimic the RGD motif (17). We also examined the effects of the monoclonal anti-GPIIb/IIIa antibody, abciximab, a chimeric mouse/human F(ab) of 7E3, which binds to the ligand binding site of GPIIb/IIIa (18). We examined the effects of the peptide RGDS and a cyclic peptide, integrin, which contains a KGD rather than an RGD sequence that is thought to improve its specificity for GPIIb/IIIa (8).

EXPERIMENTAL PROCEDURES

Reagents—Culture medium (Dulbeco’s modified Eagle’s medium/ Ham F-12), pancreatic, newborn calf serum, collagenase type II, endothelial cell growth supplement, bovine serum albumin, pancreatin, transferrin, insulin, sodium selenite, vitamin C, RGDS peptide, actived charcoal, streptavidin-fluorescein isothiocyanate, propidium iodide, monoclonal anti-α-actinin (sarcomeric) antibody (clone no. EA-53), sodium orthovanadate, Nonidet P-40, Triton X-100, glycerol, β-glycerophosphate, dithiothreitol, and common laboratory chemicals were...
from Sigma. Culture medium (M199), fetal calf serum and type II collagenase were from Worthington. Hank's balanced salt solution (HBSS), phosphate buffered saline (PBS), HEPES, penicillin G/streptomycin, l-glutamine, and sodium pyruvate were from Life Technologies Inc., Tissue culture 25-cm² flasks were obtained from Corning Costar Corporation, Cambridge, MA. Dynal, Oslo, Norway. Microtitre plates were from Amersham Pharmacia Biotech.

Amino-terminal-biotinylated peptides (bGRADSP and bGRGDSP) were from Alto Biosciences, Birmingham, UK. Aprotinin cellular-DNA fragmentation detection kit was from Roche Molecular Biochemicals. CaseACE™ Assay System Colorimetric was obtained from Promega, Southampton, UK. The Alexis caspase-3 inhibitor (al- depoxy) (Ac-DMQD-CHO) was from Cayman Chemical Company, Ann Arbor, MI. Human recombinant caspase-3 protein was from Pharmingen Division, Becton Dickinson, Oxford, UK. Orbofiban (SC57101A), xemilofiban (SC-54701A), SC52012B, and HSC52012B (19) were kind gifts from Dr. Robert Anders, J. D. Searle, Skokie, IL. Scintillation fluid, Eosicinet™ A LS-273 was from National Diagnostics, Atlanta, GA. Dynal, Oslo, Norway. Integrelin was a kind gift from Dr. David Phillips, San Francisco, UK. ABC/avidin-biotin peroxidase was from Dako Corporation, Carpinteria, CA. Protein A-Sepharose™ CL-4B beads were from Amersham Pharmacia Biotech.

Amino-terminal-biotinylated peptides (bGRADSP and bGRGDSP) were from Alto Biosciences, Birmingham, UK. Aprotinin cellular-DNA fragmentation detection ELISA™ kit was from Roche Molecular Biochemicals. CaseACE™ Assay System Colorimetric was obtained from Promega, Southampton, UK. The Alexis caspase-3 inhibitor (aldedepoxy) (Ac-DMQD-CHO) was from Cayman Chemical Company, Ann Arbor, MI. Human recombinant caspase-3 protein was from Pharmingen Division, Becton Dickinson, Oxford, UK.

Cell Culture—Primary cultures of neonatal rat cardiomyocytes were prepared by a modification of the method originally described by Simpson and Savion (31). Briefly, the hearts from 1–3-day-old Wistar rats were minced and dissociated with approximately 80 units/ml type II collagenase and 0.06% pancreatin. Cardiomyocytes were separated from other cell types by Percoll density gradient centrifugation. Dispersed cells were incubated in 25-cm² flask for 30 min at 37 °C in a CO₂ incubator. Nonattached, viable cells were collected and seeded into fresh flask at a density of 1.5 × 10⁵ cells/ml. To obtain a near pure cardiomyocyte preparation, cells were incubated in Dulbecco’s modified Eagle’s medium with Hams F-12 supplemented with 1.2 g/liter sodium hydrogen carbonate, 10% (v/v) newborn calf serum containing 1 mM pyruvic acid, 1 µg/ml transferrin, 10 µg/ml insulin, 10 ng/ml sodium selenite, 250 µM vitamin C, 100 units/ml penicillin G, and 1 µg/ml streptomycin. Using a monoclonal anti-α-actinin sarcomeric antibody we found 96–98% of our cells were cardiomyocytes (not shown). Medium was replaced with medium containing no newborn calf serum at 48 h. The cells were incubated for 24 h before the addition of the test compounds.

Coating of Streptavidin-coated Dynabeads with Biotinylated Peptides—Amino-terminal biotinylated peptides, bGRADSP and bGRGDNP, were purified by high performance liquid chromatography (by manufacturer) and were >95% pure. Streptavidin-coated Dynabeads (M-280, 2.8 µm in diameter, Dynal) were coated with biotinylated peptides according to the manufacturer’s instructions. In brief, the Dynabeads were resuspended by vortexing for 2 min, and the required volume was pipetted into a suitable tube, which was placed in the Dynal magnetic protein purification holder and allowed to settle for 2 min. The supernatant was removed carefully, and the beads were resuspended in PBS. This procedure was repeated three times. After the third wash, the beads were resuspended in the appropriate volume of PBS for coating of the beads with the biotinylated peptides. The appropriate amount of biotinylated peptides (1 µg of peptide to 10⁷ beads) was added to washed Dynabeads and incubated for 30 min at 4 °C with unidirectional mixing. The beads were collected by placing the sample tube in the Dynal magnetic protein purification holder for 2 min. The supernatant was removed, and the beads were resuspended in PBS. The washing procedure was repeated six times. The beads were then added to cells at the required concentration.

Treatment of Normal and Hypoxic Cardiomyocytes with RGDS-conjugated Biotinylated Peptides and GPIIb/IIIa Antagonists—over 24–72 h—on day 4 following isolation, medium from cardiomyocytes grown in each 25-cm² flask was replaced with fresh medium, and the compounds were added as above. The cells were then placed in a hypoxic incubator in an atmosphere of 94% nitrogen, 5% CO₂, and 1% O₂ for 48 h. Caspase-3 activity was then measured as described below.

Measurement of DNA Fragmentation—apoptosis was measured by quantitating the fragmentation of DNA in the cytoplasm of apoptotic cells. The cell lysate was placed into a streptavidin-coated microtitre plate. Subsequently, a mixture of anti-histone-biotin and anti-DNA-POD antibodies was added and incubated for 2 h. During the incubation period, the anti-histone antibody binds to the histone component of the nucleosomes and simultaneously fixes the immunocomplex to the streptavidin-coated microtitre plate. Additionally, the anti-DNA-peroxidase antibody reacts with the DNA component of the nucleosomes. After removal of unbound antibodies by a washing step, the amount of nucleosomes was quantified by the peroxidase retained in the immunocomplex. Peroxidase activity was determined photometrically with ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate)) as a substrate. A positive control was provided in the assay. Another assay was used to measure apoptosis by quantitating the fragmentation of 5'-bromo-2'-deoxyuridine-labeled DNA in apoptotic cells. The assay, a sandwich enzyme-linked immunosorbent assay (ELISA), uses two mouse monoclonal antibodies, one directed against DNA and the other against 5'-bromo-2'-deoxyuridine. Cardiomyocytes were incubated with the nonradioactive thymidine analogue 5'-bromo-2'-deoxyuridine, which is incorporated into the genomic DNA. 5'-bromo-2'-deoxyuridine fragments are released from the cells into the cell cytoplasm during apoptosis.

Measurement of Caspase-3 Activity in Cardiomyocytes—The colorimetric substrate (Ac-DEVD-pNA) was labeled with the chromophore p-nitroanilin (pNA). pNA is released from the substrate upon cleavage by DEVDase (caspase-3). The pNA produces a yellow color that was monitored by a photometer at 405 nm. The potent, irreversible, and cell-permeable pan-caspase inhibitor Z-VAD-fluoromethylketone was added directly to cells as a control. The difference between the amount of pNA produced in the absence of inhibitor and in the presence of inhibitor is largely a measure of caspase-3 and caspase-7 activity. In order to measure caspase-3 specific activity, cells were also treated with a caspase-3-specific inhibitor, Ac-DMQD-CHO. The difference between the amount of pNA released in the presence and absence of caspase-3 inhibitor minus the pNA released in the presence of the pan caspase inhibitor Z-VAD-FMK was determined as a measure of caspase-3 activity. A pNA calibration curve was plotted from a pNA stock solution provided, and caspase-3 activity in the samples was measured relative to this curve. The caspase-3 specific activity was calculated as follows, whereby:

\[ \Delta A = A_{\text{sample}} - A_{\text{control}} - A_{\text{VAD}} \]  

(1) where \( A_{\text{sample}} \) = mean induced apoptosis sample, \( A_{\text{control}} \) = mean negative control, \( A_{\text{VAD}} \) = mean negative control pan inhibitor sample, and \( A_{\text{VAD}} \) = mean blank A405. The activity (X) of caspase-3 present in each sample was calculated using the following formula.

\[ X = \text{pml of pNA liberated/h in the absence of inhibitor} \]

\[ \Delta A = \text{A} - (y \text{ intercept of pNA curve}) \times (\text{slope of pNA standard curve})^{-1} \]  

(2)

The protein concentration was determined by the method of Bradford (21). The specific activity of (SA) caspase-3 in the cell extract was calculated as follows.

Western Blot and Immunoprecipitation Analysis of Procaspase-3 Activation—Following the treatment of cardiomyocytes with RGDS-conjugated peptides and GPIIb/IIIa antagonists over 72 h, caspase-3 expression and activation was also measured directly by Western blot where the active caspase-3 was detected as its cleaved mature form using a polyclonal rabbit anti-caspase-3 antibody. In brief, for Western
blot studies, whole cell lysates were prepared with Triton X-100 buffer containing protease inhibitors. After adjustment for protein concentration, cell lysates were boiled in Laemmli buffer and resolved on 8–15% gradient SDS-polyacrylamide gel electrophoresis before Western blot analysis with an antibody against caspase-3.

Caspase-3 was purified by immunoprecipitation from lysates of cardiomyocytes in 10 mM Tris, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.5% Nonidet P-40, and 1% Triton X-100 with 20 μM caspase-3 selective inhibitor Ac-DMOD-CHO. Extracts were incubated with anti-caspase-3 antibody for 2 h at 4°C, followed by addition of the protein A-Sepharose beads. The immunoprecipitated material was washed three times in lysis buffer. The beads were solubilized in 1 ml of cell lysis buffer (10 mM Hepes, pH 7.0, 50 mM NaCl, 20% glycerol, 40 mM β-glycerophosphate, 2 mM MgCl₂, 5 mM dithiothreitol) and incubated with 1 μM peptide (GRGDSP or GRADSP) or GPIIb/IIIa antagonists; 10 μg/ml monoclonal 7E3 antibody, 10 μM integrin, 10 μM orbofiban and 10 μM xemilofiban, for 20 min at 30°C. At the end of the incubation an equal volume of Laemmli buffer was added, and the samples were boiled and resolved on 8–15% gradient SDS-polyacrylamide gel electrophoresis before Western blot analysis with an antibody against procaspase-3 and cleaved fragments.

**H-SC52021B Rapid Filteration Binding Assay**—For the competition binding assay, cardiomyocytes were incubated with: H-SC52021B for 30 min, H-SC52021B with excess SC52021B for 30 min, H-SC52021B for 72 h, H-SC52021B with excess SC52021B for 72 h, or H-SC52021B for 72 h followed by the addition of excess SC52021B over the last 30 min. Cells were washed with PBS three times and either scraped into 1 ml of Hanks/Hepes solution containing 1 mM CaCl₂ (HBSS) or lysed directly in 50 μl of 1% Triton X-100 lysis buffer on ice for 30 min. Cells in HBSS and lysis buffer were spun at 10,000 rpm in a bench top centrifuge. The cell pellet of the cardiomyocytes in HBSS was resuspended in 50 μl of HBSS. The supernatant of the lysed cardiomyocytes was removed to clean Eppendorf on ice. The cell pellet suspension in 50 μl of HBSS was passed immediately through a cell separator with a 30 μm Whatman GF/C filter to collect cell membranes. The filters were added to scintillation fluid and analyzed by scintillation counter (Wallac 1214 Rackbeta, Turku, Finland). The lysed supernatant was added directly to 5 ml of scintillation fluid and similarly analyzed. The results were plotted as counts/minute averaged over three flasks.

**Measurement of Specific H-SC52021B Binding to Human Caspase-3**—To measure specific binding of H-SC52021B to human recombinant caspase-3, 2 μg of caspase-3 reconstituted in buffer was incubated with the 20 nM H-SC52021B or 20 nM H-SC52021B in the presence of 10 μM unlabeled SC52021B or the GPIIb/IIIa antagonists orbofiban, monoclonal antibody 7E3, mAb2, or integrin for 16 h at 4°C, followed by treatment with activated charcoal to remove free radioligand. The supernatant was then counted directly. The results were plotted as counts/minute averaged over three flasks.

**Calculation of H-SC52021B Binding Affinity to Human Recombinant Caspase-3**—1 μg of human recombinant caspase-3 was incubated with increasing concentrations (6.25–1000 nM) of H-SC52021B in the presence and absence of 10 μM SC52021B for 16 h at 4°C followed by treatment with activated charcoal to remove free radioligand. The supernatant was then counted directly. The specific activity (50%) was calculated for each concentration of H-SC52021B (6.25–1000 nM), and the Kᵣ and Bᵣ values were calculated by standard saturation binding curves and Scatchard plots.

**Dual-staining Immunofluorescence and Confocal Microscopy of Cardiomyocytes Treated with Biotinylated Peptides**—Cells were incubated in the presence or absence of biotinylated peptides (GRGDSP or GRADSP) at 1 μM for 48 h. Cells were then washed in warm PBS, scraped into 1 ml of PBS, and cytospun onto glass slides. Cells were washed with fixed ice-cold acetone for 2 min followed by incubation with ice-cold methanol for 7 min followed by incubation with ice-cold acetone for 2 min. Cells were then washed in PBS for 5 min before being treated with goat serum blocking buffer for 1 h followed by treatment with rabbit-anti-rat-caspase-3 antibody at a dilution of 1/1000 for 1 h. After 1 h, cells were washed in Tris-buffered saline (3 × 5 min) and then treated with secondary goat-anti-rabbit antibody that was conjugated with a fluorescein Texas Red (Molecular Probes, Inc.). This step was followed by a washing step with 1 ml of PBS (3 × 5 min) followed by staining of the biotinylated peptides in cells with streptavidin-fluorescein isothiocyanate for 10 min. This dual-staining method allowed us to see specific binding of the biotinylated peptides to caspase-3 inside the cell. Cells were photographed using a confocal microscope (Zeiss LSM510, Axioplan 2). Pictures obtained were magnified at 40 × 10 or 63 × 10.

**Statistical Analysis**—All quantitative data are expressed as mean ± standard error. Values were analyzed using Student’s t-test. p < 0.05 was considered statistically significant.

**RESULTS**

RGD-containing Peptides and GPIIb/IIIa Antagonists Induce Apoptosis through Procaspase-3 Activation in Cardiomyocytes—RGDS showed a dose-dependent increase in apoptosis of cardiomyocytes over 24–72 h (Figs. 1 and 2) measured by either assay of DNA fragmentation, with a corresponding increase in procaspase-3 activation (Figs. 1 and 2), whereas a scrambled peptide had no effect. No increase in apoptosis was seen when RGDS was first bound to beads. The GPIIb/IIIa antagonists orbofiban and xemilofiban also caused apoptosis and procaspase-3 activation in cardiomyocytes with a similar time course (Fig. 1). In contrast, the monoclonal antibody 7E3 and the KGD-containing cyclic peptide, integrin, did not cause apoptosis in the cardiomyocytes, although some procaspase-3 activation was observed at the highest concentration of integrin after 72 h. In contrast to cardiomyocytes, orbofiban induced only a small degree of procaspase-3 activation in HUVEC and only at a very high concentration (not shown). Caspase-3 activation was also shown in cardiomyocytes by Western blot (Fig. 3A) detection of the cleaved caspase-3 p20 fragment following treatment with RGDS, orbofiban, and xemilofiban over 72 h. RGDS bound to beads; integrin and the monoclonal antibody 7E3 did not cause activation of caspase-3. Direct caspase-3 activation was shown by immunoprecipitation of procaspase-3 from cardiomyocytes followed by treatment
GPIIb/IIIa Antagonists Induce Apoptosis of Cardiomyocytes

Fig. 2. RGDS and GPIIb/IIIa antagonists cause a time-dependent increase in apoptosis and procaspase-3 activation in primary rat neonatal cardiomyocytes over 24–72 h, n = 3. A, apoptosis measured as % maximum-induced apoptosis by DNA fragmentation ELISA over 24–72 h. (**, p < 0.01; ***, p < 0.001 versus control). B, procaspase-3 activation in cardiomyocytes over 24–72 h using identical treatments (**, p < 0.01; ***, p < 0.001 versus control).

Fig. 3. Western blot detection of caspase-3 activity in cardiomyocyte whole cell lysate and in immunoprecipitated caspase-3 protein, n = 3. A, Western blot detection of caspase-3 activity in whole cardiomyocyte lysate of cells treated with (lanes 1, 2), RGDS bound to beads; lane 3, 10 μM integrin; lane 4, 10 μg/ml monoclonal 7E3 antibody; lane 5, 600 μM RGDS; lane 6, 10 μM orbofiban; lane 7, 10 μM xemilofiban, over 72 h (lane 1, molecular weight markers). B, Western blot detection of caspase-3 activity in immunoprecipitated procaspase-3 protein which was treated as above (lanes 1–7), over 30 min at 30 °C.

with the compounds (Fig. 3B). RGDS, orbofiban, and xemilofiban caused direct activation of caspase-3 measured by Western blot. Interestingly the p20-cleaved fragment was also detected in the monoclonal antibody 7E3-treated protein, suggesting that this compound must enter the cell to cause activation of procaspase-3. Neither RGDS bound to beads nor integrin caused direct activation of caspase-3.

Fig. 4. 3H-SC52012B, a GPIIb/IIIa antagonist binds to cytosolic protein in cardiomyocytes after 72 h, n = 3. Primary rat neonatal cardiomyocytes were incubated with 20 nM 3H-SC52012B alone or 20 nM SC52012B in the presence of 10 μM SC52012B for 30 min or 72 h before harvesting cells. There was no specific membrane or cytoplasmic binding after 30 min. After 72 h there was no membrane binding; however, there was a significant increase (***, p < 0.001 versus 30-min binding) in cytosolic binding that was suppressed by excess SC52012B ($$, p < 0.001 versus cytosolic bound ligand).

GPIIb/IIIa Antagonist 3H-SC52021B Enters the Cell and Binds to a Cytoplasmic Protein—Following incubation of cardiomyocytes with 3H-SC52021B for 30 min, no significant binding to cardiomyocytes was found (Fig. 4). After 72 h, however, there was a marked increase in binding confined largely to the cytosolic fraction and not the membrane fraction. The cytosolic labeled ligand was competed off by unlabeled SC-52021B added at the start of the 72-h incubation and not when added for the last 30 min, consistent with a gradual uptake of the labeled compound over the period of incubation.

3H-SC52021B Binding to Human Recombinant Caspase-3—3H-SC52021B bound to human recombinant caspase-3 with a Kd of 59 ± 2 nM and a Bmax of 324 ± 32 fmol/μg of protein (n = 3). 3H-SC52021B binding to human caspase-3 (Fig. 5) was competed by unlabeled SC-52021B, orbofiban, xemilofiban, and by the monoclonal 7E3 antibody. In contrast, integrin did not compete for binding. Similarly mAb2, a murine GPIIb/IIIa monoclonal antibody that does not bind to the ligand binding site, had no effect on 3H-SC52021B binding to caspase-3. As a control experiment, we measured the binding of 3H-SC52021B to milk protein under the same conditions and examined the effects of the compounds. 3H-SC52021B bound nonspecifically to this protein in that neither unlabeled SC-52021B nor the other GPIIb/IIIa antagonists inhibited binding.

Dual-staining Immunofluorescent Confocal Microscopy Revealed Co-localization of RGDS-biotinylated Peptide to Procaspase-3—Cells were stained with peptide (green) and an antibody to caspase-3 (red). GRGDSP (Fig. 6C) but not GRGDS (Fig. 6F) biotinylated peptides bound to procaspase-3 inside the cardiomyocyte (co-localization was stained white).

Orbofiban Shows Enhanced Potency against Hypoxic Cardiomyocytes—Hypoxia (1% O2) caused a significant increase in apoptosis and procaspase-3 activity in cardiomyocytes after 48 h (Fig. 7). The degree of apoptosis (not shown) and procaspase-3 activation was markedly potentiated by orbofiban even at a concentration as low as 10 nM. The degree of procaspase-3 activation far exceeded that seen in normoxic cells. Neither integrin nor the monoclonal 7E3 antibody increased the hypoxic-mediated procaspase-3 activation.
RGD is a common integrin recognition sequence in adhesive proteins, and cardiomyocytes exhibit anchorage-dependent growth. Therefore, a possible explanation for these findings is interruption of integrin-mediated signaling or cell-cell interactions required for cell survival (22). To address this possibility, we examined the effects of an RGDS-containing peptide (GRGDSP) bound to beads. No apoptosis was seen with the RGDS-coated beads. However, the addition of soluble GRGDSP to this preparation induced apoptosis (not shown).

These results indicated that RGDS peptides must enter cardiomyocytes to induce apoptosis. Indeed, the labeled GPIIb/IIIa antagonist \(^{3}H\)-SC52012B did not bind to cardiomyocyte cell membranes over 30 min. However, there was a time-dependent increase in \(^{3}H\)-SC52012B within the cytosolic fraction that peaked at 72 h. In the absence of any specific cell binding, it is unlikely that the uptake of the drug involved a specific protein or transport system. These results suggest that the compounds enter by passive diffusion, as shown previously for RGD-peptides (9). These compounds are highly charged and may act as zwitterions, so that at the lower pH of the cell, they are retained.

Caspases, derived from their precursor and catalytically inactive procaspases, are a series of degradative enzymes that are final products of programmed cell death (10). Caspase-3 has been found to mediate the apoptosis that occurs in response to ischemia and doxorubicin in cardiac cells (15). Caspases contain an RGD motif and in some cases an RGD recognition site that may control protein folding and activation. Caspase-1 contains an RGD motif next to the catalytically active cysteine residue (QAR286GDSP) and a homologous single RGD sequence is found in caspase-2 (QACR304GD) (9). The large subunit of caspase-3 and caspase-7 and the small subunit of caspase-8 also contain an RGD sequence. RGD-binding motifs found in integrins (D315DA and D180DM) (23) are also found in procaspase-1 and -3, respectively close to experimentally determined processing sites. RGD-containing peptides activate procaspase possibly by inducing conformational changes resulting in the oligomerization and subsequent autoprocessing required for activation. Rat procaspase-3 also contains both an R144GD sequence and a potential RGD-binding site (D180DM) next to the catalytically active cysteine residue (QAR286GDSP). RGDs, orbofiban, and xemilofiban increased caspase-3 activity in the rat cardiomyocytes after 48–72 h in parallel with the increase in apoptosis. In contrast, the GPIIb/IIIa antagonist 7E3, a monoclonal antibody, did not cause significant caspase-3 activation potentially as the antibody cannot cross the cell membrane. Integrin at high concentrations induced a small increase in procaspase-3 activation but to a far lesser extent than seen with either orbofiban or xemilofiban and at a concentration that exceeds by orders of magnitude the concentration required to prevent platelet aggregation (24). Integrin is a cyclic peptide containing a KGD rather than an RGD sequence (19). The lack of effect of integrin on procaspase-3 may reflect the strict peptide sequence requirement for activation of procaspase-3 (9). When procaspase-3 was immunoprecipitated from cardiomyocytes RGDs, orbofiban, xemilofiban, and 7E3 directly activated procaspase-3, whereas integrin had no effect. RGD-containing GPIIb/IIIa antagonists activated procaspase-3 directly, whereas non-RGD-containing antagonists like integrin had no effect. It is possible that the caspases are involved in inducing cell death in cardiomyocytes, including caspase-7, which is also detected in our assay of DEVDase activity.

As procaspase-3 contains an RGD recognition sequence (DDM), we next assessed whether \(^{3}H\)-SC52012B could bind directly to recombinant human caspase-3 protein using a char-

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**DISCUSSION**

GPIIb/IIIa antagonists are often RGD-mimetics designed to inhibit platelet aggregation by preventing fibrinogen binding to the integrin, \(\alpha_{IIb}\beta_{IIIa}\). Paradoxically, one of these (orbofiban) has been reported to increase mortality in patients with coronary artery disease. Peptides containing an RGD motif induce apoptosis in resting (G0) peripheral blood T cells (9). Consequently, we investigated the effects of an RGDS peptide in inducing apoptosis measured by DNA fragmentation in primary rat cardiomyocytes. RGDS peptide caused a dose-dependent increase in apoptosis over 24–72 h with half-maximal effect occurring at 300 \(\mu\)M after 72 h, whereas the scrambled peptide GRADSP had no effect. Two RGD-mimetics, orbofiban and xemilofiban that have a high degree of specificity for platelet GPIIb/IIIa (17) also caused dose-dependent apoptosis of cardiomyocytes over 24–72 h. In contrast, the monoclonal antibody 7E3, which is directed against the ligand binding site of GPIIb/IIIa, and the cyclic heptapeptide integrin, a GPIIb/IIIa antagonist that contains a KGD rather than an RGD sequence, did not induce significant apoptosis. The effect of orbofiban was cell-specific in that it had little effect on HUVEC.
coal adsorption technique for separating bound and free radioligand. ³H-SC52012B bound to human caspase-3 to a greater extent than to a control milk protein. The addition of unlabeled SC52012B reduced the binding to that seen with the control protein (nonspecific binding to protein or charcoal) indicating that binding to caspase-3 was specific. In contrast, unlabeled SC52012B had no effect on ³H-SC52012B binding to the control protein. The $K_d$ for binding to caspase-3 is similar to the $K_d$ for human GPIIb/IIIa (17). ³H-SC52012B was displaced by orbofiban and by the monoclonal antibody 7E3. As 7E3 did not induce cell death, this finding further supports the hypothesis that the compounds must gain access to cells in order to activate cell death pathways. In contrast mAb2, an antibody to an epitope on GPIIb/IIIa remote from the ligand binding site (25), had no effect. Similarly, integrin at 10 μM did not compete with ³H-SC52012B binding, consistent with the absence of an effect on apoptosis and procaspase-3 activation. There is no evidence of widespread tissue damage in patients receiving GPIIb/IIIa antagonists. Indeed, there was no procaspase-3 activation in HUVEC and no evidence of apoptosis (data not shown). Moreover, the plasma concentrations of orbofiban and xemilofiban inducing apoptosis in cardiomyocytes are far in excess of those achieved at doses used to inhibit platelet aggregation (200–400 nm) (26). However, the target population for GPIIb/IIIa antagonists (patients with coronary artery disease) may have or may develop cardiac hypoxia as a result of coronary artery obstruction. Hypoxia has been shown to trigger cell death through activation of procaspase-3 (27, 28). Consequently, we examined whether stressed cardiomyocytes were more susceptible to the effects of GPIIb/IIIa antagonists. Orbofiban at a concentration as low as 10 nM induced apoptosis and activated procaspase-3 in cardiomyocytes grown in 1% O₂. Moreover, the degree of apoptosis and enzyme activation exceeded that seen in cardiomyocytes grown under normoxic conditions. Again, neither integrin nor the monoclonal 7E3 antibody increased procaspase-3 activation in hypoxic cardiomyocytes. It is possible that the hypoxic cells are more susceptible to orbofiban due to hypoxia.
increased membrane permeability or due to co-activation of procaspase-3 by hypoxia and the GPIIb/IIIa antagonist.

Numerous studies have shown that the disruption of cell-matrix interactions leads to apoptosis in anchorage-dependent cells (11), possibly because of a loss of adhesion-mediated survival signals (12, 22). Consequently, peptides and nonpeptide mimetics based on the RGD motif have been used extensively to inhibit tumor metastasis (11), abrogate T-cell-mediated immune responses in vivo (13), induce endothelial cell apoptosis in models of angiogenesis (12), and inhibit inflammation (29). Perhaps RGD peptides induce apoptosis through direct caspase-3 activation and therefore may be capable of causing apoptosis in many cell types independent of their interactions with integrins.

The phase III clinical trial of orbofiban, OPUS-TIMI16, was halted recently due to an increase in mortality, largely cardiac in origin. In part, this may have reflected a paradoxical activation of platelets as has been reported for several of these compounds. Interestingly, Shcherbina and Remold-O'Donnell (30) have reported the presence of caspase-3 in human platelets and its activation by platelet agonists. Our results provide an alternate explanation, that under conditions of ischemia or infarction orbofiban contributed to the associated cardiac damage. It is worth noting that the Swissprot data base identifies several proteins, in addition to procaspase-3, that contain both an RGD motif and RGD-binding site and that are important for cardiac function. One of these, the ryanodine receptor controls calcium release from the sarcoplasmic reticulum. Other cardiac proteins with both motifs include transcriptional endoplasmic reticulum ATPase, cytochrome b_{5}, the prostaglandin F_{2alpha} receptor-regulating protein, and myosin. Therefore, the biological activity of RGD peptides and nonpeptide RGD mimetics may be broader than simple interruption of adhesion.

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Glycoprotein IIb/IIIa Antagonists Induce Apoptosis in Rat Cardiomyocytes by Caspase-3 Activation
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