α1-Proteinase Inhibitor, α1-Antichymotrypsin, and α2-Macroglobulin Are the Antiapoptotic Factors of Vascular Smooth Muscle Cells*

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Serum depletion induces cell death. Whereas serum contains growth factors and adhesion molecules that are important for survival, serum is also likely to have antiapoptotic factor(s). We show here that the plasma proteinase inhibitors α1-proteinase inhibitor, α1-antichymotrypsin, and α2-macroglobulin function as critical antiapoptotic factors for human vascular smooth muscle cells. Cell survival was assured when serum-free medium was supplemented with any one or all of the above serine proteinase inhibitors. In contrast, the cells were sensitive to apoptosis when cultured in medium containing serum from which the proteinase inhibitors were removed. The antiapoptotic effect conferred by the proteinase inhibitors was proportional to proteinase inhibitory activity. Without proteinase inhibitors, the extracellular matrix was degraded, and cells could not attach to the matrix. Cell survival was dependent on the intact extracellular matrix. In the presence of the caspase inhibitor z-VAD, the cells detached but did not die. The activity of caspasas was elevated without proteinase inhibitors; in contrast, caspasases were not activated when medium was supplemented with one of the proteinase inhibitors. In conclusion, the plasma proteinase inhibitors prevent degradation of extracellular matrix proteins by cell-derived proteases. Thus, the plasma proteinase inhibitors are essential to ensure appropriate cell-matrix interactions. Analysis of the cells that detach in the absence of proteinase inhibitors showed a similarity to apoptotic cells induced by serum depletion. Based on these results, we hypothesized that the proteinase inhibitors function as critical survival factors in vascular smooth muscle cells.

EXPERIMENTAL PROCEDURES

Induction of Cell Death—Cultured human vascular smooth muscle cells (HNB1E6E6E7) were originally derived from the aorta of a newborn infant (2-day-old) autopsy (11, 12). The cells were detached by brief exposure to 0.05% trypsin, 0.02% EDTA. Trypsin was inactivated by excess amounts of soybean trypsin inhibitor (Sigma). After washing with PBS, the cells were resuspended in serum-free DMEM containing 0.2% bovine serum albumin, at a concentration of 50,000 cells/50 μl. A 96-well culture plate was previously coated with either fibronectin (10 μg/ml) or vitronectin (10 μg/ml) at room temperature for 1 h. The cell suspensions (50 μl) were plated on the 96-well culture plate. 50 μl of each sample were overlaid. Final concentration of the samples was 5% calf serum, PDGF-BB (20 ng/ml), α1PI (0.125 mg/ml), α1ACT (0.125 mg/ml), and α2M (0.125 mg/ml) in DMEM with 0.2% bovine serum albumin. The plates were incubated at 37°C with 5% CO2 for 24 h. Attached cells were counted using phase contrast microscopy. Fibronectin, vitronectin, α1PI, α1ACT, and α2M were purchased from CalBiochem. PDGF-BB was from Life Technologies, Inc. The experiments were repeated three times.

Western Blot Analysis—After observing cell morphology, 25 μl of 25% SDS was added to the wells and shaken for 1 h. The samples were run on an 8% SDS-polyacrylamide gel electrophoresis, and proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad). The membrane was blotted with polyclonal anti-fibronectin antibody (B790), a kind gift provided by Dr. William G. Carter (Fred Hutchinson Cancer Research Institute, Seattle, WA), at 1:10,000 dilution, and the antigen was visualized with Renaissance Western blot chemiluminescence reagent (PerkinElmer Life Sciences).

Trypan Blue Exclusion Assay—After collecting floating cells, attached cells were exposed to 0.05% trypsin, 0.02% EDTA. Trypsin was inactivated by soybean trypsin inhibitor (Sigma). All the attached and detached cell populations were combined to determine the proportion of dead cells. Trypan blue (Life Technologies, Inc.) was mixed with cells (1:1), and trypan blue exclusion by living cells was scored using phase contrast microscopy.

TUNEL Staining—TUNEL staining was performed using the in situ cell Death Detection Kit, Fluorescein (Roche Molecular Biochemicals cat. No. 1 684 795). The nuclei were counterstained with Hoechst at 5 μM.

Inactivation of α1PI and α2M—α1PI was inactivated according to Johnson and Travis (13); 0.1 mg of human α1PI (CalBiochem) in 40 μl

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1 The abbreviations used are: α1PI, α1-proteinase inhibitor; α1ACT, α1-antichymotrypsin; α2M, α2-macroglobulin; PDGF, platelet-derived growth factor; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; z-VAD, benzylxoycaryonyl-VAD; TUNEL, terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick-end labeling.

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of 0.1 M Tris buffer, pH 8.8 was mixed with 10 μl of 0.8 M N-chloro-
succinimide (Sigma) and incubated overnight. The excess of the reagent
was removed by dialysis. The remaining antitrypsin activity was less
than 24 h. N and PDGF were significantly lower than the other four
samples, p < 0.001. B, photographs were taken at 24 h with a phase contrast
microscope. Previous coat on plastic surface was either fibronectin (FN)
or vitronectin (VN) (original magnification, ×100). C, Western blot analysis for fi-
bronectin. After observation of cell morphology as shown in B, the coated fi-
bronectin was solubilized with 5% SDS. Each sample was electrophoresed on 8% SDS-polyacrylamide gels, transferred to a poliviniliden difluoride membrane, and blotted with a polyclonal antibody against fi-
bronectin.

FIG. 1. Cell attachment on fibronectin. A, vascular smooth muscle cells were
cultured on a plastic surface previously coated with fibronectin. Culture medium
was DMEM with 0.2% bovine serum albumin, pH 7.4, containing either 5% calf
serum (CS), no serum (N), platelet-derived growth factor (PDGF) at 20 ng/ml, α1-proteinase
inhibitor (PI), α1-antichymotrypsin (ACT), and α2-macroglobulin (M) at 0.125 mg/ml. Attached cell num-
bers were counted using a phase contrast microscope at 1 h and 24 h. N and PDGF
were significantly lower than the other four groups, p < 0.001. B, photographs
were taken at 24 h with a phase contrast microscope. Previous coat on plastic sur-
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ferred to a poliviniliden difluoride membrane, and blotted with a polyclonal antibody against fi-
bronectin.

RESULTS
Human vascular smooth muscle cells (HNB18E6E7) were
plated in DMEM medium containing either calf serum, PDGF-
BB, α1PI, α1ACT, or α2M on 96-well culture plate previously
coated with either fibronectin or vitronectin. PDGF-BB induces
cell division of HNB18E6E7 cells as shown by increased thy-
midine incorporation (data not shown). Attached cells were
counted using a phase contrast microscope. At 1 h, cell attach-
ment on the surface was similar. However, at 24 h, the num-
bers of attached cells were significantly different (Fig. 1A).
Fig. 1B shows photographs at 24 h, when cells had detached and
looked round upon serum depletion or in the presence of PDGF-
BB. In contrast, with serum, α1PI, α1ACT, or α2M, cells re-
main attached and spread. A number of cells with calf serum
was about 10% more than α1PI, α1ACT, or α2M, probably
because the growth factors in serum supported proliferation.

To determine whether the endogenous cellular proteinases
disrupted the cell matrix, we examined the integrity of fibro-
nectin. Specifically, fibronectin was extracted by 5% SDS, and then
Western blot analysis was performed. Fibronectin was intact
with serum, α1PI, α1ACT, or α2M; however, it was degraded
with PDGF or no serum (Fig. 1C). Therefore, the morphology
of the cells depended on the integrity of adhesion molecules.

To determine how many cells were dead, we collected all the
detached and attached cells and then counted the total number
of dead cells by trypan blue exclusion test (Fig. 2A). Dead cell
numbers were significantly higher with no serum or PDGF
alone than with serum or proteinase inhibitors. Next, we con-
firmed apoptosis using TUNEL staining although TUNEL de-
ects only of attached cells (Fig. 2B). TUNEL positivity
was significantly higher with no serum or PDGF than other
groups. These data suggest that proteinase inhibitors prevent
degradation of matrix, which supports cell attachment and
may result in cell survival.

Next, we quantified the survival effect of the proteinase
inhibitors, α1PI, α1ACT, and α2M induce cell survival in a
dose-dependent manner by the trypan blue exclusion test (Fig.
3A). In this assay, 50% survival was achieved at 4.4 × 10 −8 M
in α2M, at 3.3 × 10 −7 M in α1PI, and at 4.2 × 10 −7 M in α1ACT.
Thus, α2M has a 7.6 times higher ability than α1PI and 9.6
times higher than α1ACT. We next removed both α1PI and
α2M from human serum by immunoprecipitation. We did not
have an appropriate antibody against α1ACT and thus could
not deplete it. Ninety-five percent of the proteinase inhibitory activity was lost by the simultaneous removal of both proteins. The remaining 5% activity was probably due to the presence of 1ACT. The immunoprecipitated serum lost 95% of survival activity, assessed by trypan blue (Fig. 3B). Furthermore, we tried an admixture of three proteins at normal serum level. Survival rate with the admixture was not statistically different from that with serum in this assay (Fig. 3B).

Chemical inactivation was performed on 1PI and 2M. 1PI was inactivated with N-chlorosuccinimide. 2M was inactivated with methylamine. Both 1PI and 2M lost their survival effect (Fig. 3C). These results indicate that the ability of cell survival is attributable to proteinase inhibitory activity.

We compared the proteinase inhibitors with a caspase inhibitor as survival factors. Cells attached on a plastic surface previously coated with fibronectin at 24 h with 2M (Fig. 4A); however, cells detached with no serum (Fig. 4B) or with z-VAD (Fig. 4C). The level of attached cells was significantly higher with 2M than other groups (Fig. 4D). In contrast, with z-VAD, the cells are alive (assessed by trypan blue exclusion) although many cells have detached (Fig. 4E). z-VAD had no effect on attachment although detached cells survived with z-VAD.

Next, we determined caspase activity using caspase-specific fluorescent substrate, PhiPhiLux-G1D2. With 2M, caspase activity was as low as with z-VAD (Fig. 5). However, without 2M, caspase activity was significantly high, similar to that with staurosporin. This suggests that the proteinase inhibitor not only supports attachment but also prevents activation of caspasas.

The data suggest a novel mechanism for cell survival induced by plasma proteinase inhibitors. Cell survival is dependent on the ability of proteinase inhibitor to protect adhesion matrix. If the proteinase inhibitors are not present, the matrix is degraded by cell-derived proteinase(s).

DISCUSSION

It is well documented that extracellular matrix and integrin interactions generate intracellular signals that lead to caspase activation (14–16). However, combinations of adhesion molecules and growth factors alone are often insufficient to keep cells in culture alive without serum. Serum therefore, has been assumed to include unidentified survival factor(s). We have demonstrated that the critical antiapoptotic factors in serum are 1PI, 1ACT, and 2M. We also show that the mechanism of survival involves proteinase inhibitors, protecting the extracellular matrix from being degraded by cell-derived proteinase(s).

Cell migration is required during development, in tissue repair and in wound healing. Degradation of the extracellularmatrix is a critical process for cell migration. Proteinase inhibitors are known to protect extracellular matrix from degradation by proteinases. Our results suggest that proteinase inhibitors may play a role in protecting extracellular matrix from degradation by proteinases, thus allowing cell migration.

FIG. 2. **Quantification of dead cells.** A, all the detached and attached cells were collected after observation of cell morphology as shown in Fig. 1. Dead cells were counted using trypan blue exclusion. The error bars represent S.D.; asterisk represents p < 0.05 compared with the no serum group. B, TUNEL staining for the attached cells shown in Fig. 1. The attached cells were double stained with TUNEL and Hoechst. Positivity was counted using a fluorescent microscope. The error bars represent S.D.; asterisk represents p < 0.05 compared with the no serum group.

FIG. 3. **Survival effect of proteinase inhibitors.** A, trypan blue exclusion was performed for all the attached and detached cells. Each proteinase inhibitor worked as a survival factor in a dose-dependent manner. B, 1PI and 2M were subtracted from normal human serum by immunoprecipitation (serum-PI). PBS indicates a negative control. PBS+PI indicates an admixture of the three proteinase inhibitors at normal serum level. Final concentration was equal to 2.5% serum in each well. The error bars represent S.D.; asterisk indicates p < 0.01 compared with negative control. C, 1PI inactivated by N-succinichloroamide (PI(-)) and native 1PI were compared at each concentration. 2M inactivated by methylamine (M(-)) and native 2M were compared at each concentration. Death was quantified by trypan blue exclusion.
matrix is a prerequisite for cell migration into the three-dimensional matrix. As part of this homeostatic process, cells produce proteinases, including matrix metalloproteinases (17) and/or serine proteinases. Careful regulation of proteolytic activity is required, as too much degradation will induce detachment that may result in cell death. In many cases, matrix proteinase activity is immediately neutralized. In addition to plasma proteinase inhibitors, many proteinase inhibitors such as tissue inhibitor of matrix metalloproteinases (TIMPS) have activity against specific cell-derived proteinases (18). Because a broad spectrum of proteases have apoptotic activity, it is reasonable to assume that many proteinase inhibitors are potentially antiapoptotic factors, acting via a mechanism similar to what we have shown here.

There is some controversy over whether TIMPs are proapoptotic or antiapoptotic. TIMP-3 induces apoptosis (19–22) via a death domain located within the N terminus (23). Alexander et al. (24) showed that overexpression of TIMP-1 prevents apoptosis of epithelial cells from stromelysin-1 transgenic mice. Several other studies have shown antiapoptotic effects of TIMP-1 (25, 26) and TIMP-2 (27). The role of proteinases is not limited to matrix degradation in vivo. For example, extracellular proteinases can directly modify intracellular signals via proteinase-activated receptors (28). Thus, the interaction between proteinases and proteinase inhibitors may influence a cell's response to its environment in several ways.

Compared with the abundance of albumin and globulin, proteinase inhibitors are the third most prevalent group of plasma proteins. In our assay, neither albumin nor globulin demonstrated any antiapoptotic effect whereas α2M, a very nonspecific protease inhibitor, was very cytoprotective. α2M is an ancient protein that is found in all vertebrates and in non-vertebrates such as eels and crabs (29). There is speculation that α2M is a carrier of transforming growth factor-β and other cytokines (30) or that it functions as an agonist of α2M receptors (31). No α2M genetic deficiency has been found in our species despite extensive screening of human plasma (32). Furthermore, α2M knock-out mice have no phenotype (33). α1PI is a universal inhibitor of serine proteinases, which when diminished or mutated causes pulmonary emphysema and liver fi-
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Fig. 5. Caspase activity was determined using fluorescent substrate PhiPhiLux-G1D2 (see “Experimental Procedures”). Human vascular smooth muscle cells were cultured on a plate previously coated with fibronectin with α2M at $1.7 \times 10^{-7}$ M (A), without α2M (B), with z-VAD at $1 \times 10^{-4}$ M (C) or with stauros- porin at $1 \times 10^{-6}$ M (D). Intensity of fluorescence was measured using cell sorter. Mean of PhiPhiLux activity (E) and percent of cell numbers in a gait (M1) were plotted (F). St represents stauros- porin. Each experiment was repeated three times. Asterisk represents $p < 0.05$ compared with α2M(−).

brosis. Physiologically, α1PI is considered to be an elastase inhibitor (32). α1ACT is a chymotrypsin type serine proteinase inhibitor. α1ACT is recognized as an acute phase protein. Serum concentrations of α1ACT increase rapidly and dramatically after a variety of events including surgery, burn injury, inflammatory bowel disease, and some types of cancer (32). This probably indicates that an increase in α1ACT supports the survival of resident cells in areas of inflammation. Although the actual roles of the plasma proteinase inhibitors are not completely clarified, our data show a novel biological activity of these proteinase inhibitors.

One obvious question is what proteinase(s) does the smooth muscle cell produce? Based on the specificity of proteinase inhibitors, chymotrypsin type serine proteinases are candidates. We observed that this cell line produced chymotryptic activity when co-cultured with a chymogenic substrate (data not shown). Additional experiments using degenerate PCR primers and screening of a large gene expression array also identified a trypsin-like protease (manuscript in preparation).

In conclusion, we have shown a novel anti-apoptotic activity of plasma proteinase inhibitors. Because of the high concentration of α1PI and α2M, the proteinase inhibitors may function as the major antiapoptotic proteins present in serum. The proteinase inhibitors prevent extracellular matrix from degradation by cell-derived proteinase(s). This novel activity may suggest that the proteinase inhibitors could play a role in vascular disease such as atherosclerosis or aortic aneurysm.

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