Possible Involvement of m-Calpain in Vascular Smooth Muscle Cell Proliferation

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Abstract—Vascular smooth muscle cell (VSMC) proliferation still remains a poorly understood process, although it is believed to play a critical role in pathological states, including atherosclerosis and hypertension. Several reports have suggested that proteases may be directly involved in this process; however, it was still unclear which protease is responsible for VSMC proliferation. In this study, by use of a cell-permeable calpain inhibitor (calpeptin; benzylxoycarbonyl-Leu-nLeu-H), its analogue (benzylxoycarbonyl-Leu-Met-H), the cell-impermeable serine protease inhibitor leupeptin, and antisense oligonucleotide against m-calpain to inhibit proliferation of primarily cultured human VSMCs, we investigated whether calcium-activated neutral protease (calpain) is involved in VSMC proliferation. Calpeptin and its analogue, more specific for m-calpain, equally inhibited the proliferation of VSMCs in a dose-related manner, whereas a more limited antiproliferative effect was observed in leupeptin-treated VSMCs. Antisense oligonucleotide against m-calpain, but not scrambled antisense, dose-dependently inhibited m-calpain expression and proliferation of VSMCs. Maximal inhibition was an ≈50% reduction of cell number and m-calpain antigen observed at 50 μmol/L of antisense oligonucleotide. Calpeptin or antisense oligonucleotide against m-calpain increased the expression of the endogenous calpain substrate pp125FAK (focal adhesion kinase), whereas the expression of the endogenous calpain inhibitor calpastatin was not affected. These results suggest that the proliferation of VSMCs requires protease activity, some of which is due to m-calpain.

Key Words: calpain ■ vascular smooth muscle cells ■ proliferation

Several reports have suggested the role of [Ca^{2+}], in regulating transitions of the cell cycle, not only in mitosis but also in the G1-to-S transition. Although the phenomenon of a rise in [Ca^{2+}], and the mechanisms to induce this rise have been intensively studied, the mode of cell cycle regulation by [Ca^{2+}], has been poorly documented. It is likely that the [Ca^{2+}],-dependent signal transduction pathway, including calmodulin–dependent reactions, the protein kinase C–mediated pathway, and the calcium-activated neutral protease (calpain)–mediated pathway, plays an important role as an effector for elevated [Ca^{2+}], but the exact mechanisms integral to cell proliferation are still unclear.

Calpains (EC 3.4.22.17) are Ca^{2+}-requiring intracellular cysteine proteases that are ubiquitously distributed in mammalian and avian cells. There are at least two major calpain isoforms: m-calpain, which requires millimolar Ca^{2+} for its activation, and μ-calpain, which requires 10 to 100 μmol/L Ca^{2+}. There are also tissue-specific forms of calpain, and most cells contain an endogenous inhibitor protein, calpastatin, which is specific for the calpains. Although their exact physiological function has not been established, a variety of substrate proteins, including enzymes, surface glycoproteins, and cytoskeletal proteins, have suggested the possible involvement of this enzyme-inhibitor system in Ca^{2+}-dependent signal transduction pathways.

Earlier studies using protease inhibitors have suggested the involvement of protease activity in vascular smooth muscle cell proliferation. This hypothesis has been strengthened by the observations in other cell types; Schollmeyer reported the acceleration of anaphase by microinjection of m-calpain in PtK1 cells, Zhang et al reported the growth inhibition of HeLa and WI-38 cells induced by treatment with a calpain small-subunit antisense oligonucleotide, and Mellgren et al observed reduced proliferation of Chinese hamster ovary cells with decreased μ-calpain content after treatment with calpain inhibitors. Although these observations strongly suggested the roles of calpains in VSMC proliferation, the exact relationships between proteolytic activity of calpain and VSMC proliferation is still unclear, partly because of a lack of knowledge about calpain activity without a small subunit or the exact inhibitory spectra of protease inhibitors. In this study, we used several protease inhibitors with different inhibitory characteristics and an antisense oligonucleotide against a large subunit of m-calpain to clarify the exact roles of m-calpain in VSMC proliferation.

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Methods

Materials
Primary human vascular smooth muscle cells (VSMCs) were prepared from human gastroduodenal artery as previously outlined and were used in the third passage.31 Cells were cultured in DMEM supplemented with 10% FBS (10% FBS-DMEM). The characteristics of monoclonal antibodies specific for \( \mu \)-calpain (1A8A2), m-calpain (1C6D1), and calpastatin are described elsewhere.32 Monoclonal antibody specific for pp125FAK was purchased from Transduction Laboratories.24 FITC-conjugated rabbit anti-mouse IgG (secondary antibody) was purchased from Cappel. Purified antisense phosphorothioate oligonucleotides were synthesized by Vector Research. The sequence for the antisense m-calpain oligonucleotide was CGCGATGCCCGCCCGCCATGCT. A corresponding scrambled sequence (GGCTGCCGCGAGCCCCACTCT) was used as control. Calpeptin (benzyloxy carbonyl-Leu-nLeu-H) and its analogue (benzyloxy carbonyl-Leu-Met-H) were synthesized as we described previously.33 Other reagents were of the highest analytical grade available.

Growth Assay
VSMCs were seeded in six-well plates in 10% FBS-DMEM. The following day, the cells were washed twice with PBS, and the medium was replaced with 0.5% FBS-DMEM (growth-arrest medium). The cells were kept in growth-arrest medium for 96 hours for synchronization to minimize artifacts due to the heterogeneous cell-cycle stage during the growth assay. The medium was then changed to 10% FBS-DMEM, and several protease inhibitors or synthetic oligonucleotides were added. The cells were permitted to grow for 72 hours and then trypsinized and counted on a Coulter counter.

Immunofluorescence Microscopic Examination of VSMCs for Calpains and Calpastatin
VSMCs were fixed with 2% paraformaldehyde/PBS at room temperature for 15 minutes, permeated with 0.2% Triton X-100/PBS, washed three times with 1% BSA/PBS, and exposed for 2 hours to anti-\( \mu \)-calpain, anti-m-calpain, or anti-calpastatin monoclonal antibody diluted to 5 \( \mu \)g/mL in 1% BSA/PBS. The cells were washed three times with 1% BSA/PBS to remove excess primary antibody, followed by incubation for 2 hours with secondary antibodies diluted 1:100 in 1% BSA/PBS. After the cells were washed three times with 1% BSA/PBS, they were examined by confocal laser scanning microscopy as described in “Methods.” Results shown are from one representative of four different experiments.

Results
Expression of \( \mu \)-Calpain, m-Calpain, and Calpastatin in Primary VSMCs
To confirm the presence of the calpain-calpastatin system in human VSMCs, we first examined the immunoreactivity of \( \mu \)-calpain, m-calpain, and calpastatin by immunofluorescent staining using specific monoclonal antibodies.32 As shown in Fig 1, VSMCs showed strong immunoreactivity to anti-\( \mu \)-calpain and anti-calpastatin monoclonal antibodies but failed to show clear immunoreactivity to anti-m-calpain monoclonal
antibody. Although these observations cannot exclude the existence of μ-calpain or suggest a lower expression of calpastatin than of m-calpain, they clearly confirm the existence of m-calpain and calpastatin in VSMCs. Because of the lack of methods to detect the m-calpain molecule in VSMCs, we tested the effect of antisense oligonucleotide against m-calpain in further studies.

**Influence of m-Calpain Antisense Oligonucleotide on the Immunoreactivity of m-Calpain in VSMCs**

As shown in Fig 2, treatment with antisense oligonucleotide against m-calpain caused a marked decrease in m-calpain detected by immunofluorescent technique, whereas control oligonucleotide did not cause a remarkable decrease in m-calpain in VSMCs.

**Influence of m-Calpain Antisense Oligonucleotide or Calpeptin on the Immunoreactivity of m-Calpain, Calpastatin, and pp125FAK in VSMCs**

The effect of antisense oligonucleotide against m-calpain was further confirmed by Western blot analysis, as shown in Fig 3A and 3B. Although neither control oligonucleotide nor the cell-permeable calpain inhibitor calpeptin caused any change in m-calpain, m-calpain antisense oligonucleotide caused a significant decrease in m-calpain in a dose-related manner in VSMCs. Maximal decrease of m-calpain was observed at 50 μmol/L antisense oligonucleotide. To estimate the activity of calpain in VSMCs treated with m-calpain antisense oligonucleotide or calpeptin, the expression of the endogenous calpain inhibitor calpastatin or the endogenous calpain substrate pp125FAK was also examined. As shown in Fig 3B, calpeptin as well as m-calpain antisense oligonucleotide increased pp125FAK, whereas calpastatin was not affected. Although we failed to detect the cleavage products of pp125FAK, this may be a result of the long incubation time for the growth assay.

**Effects of Protease Inhibitors and m-Calpain Antisense Oligonucleotide on VSMC Growth**

As shown in Fig 4, a dose-dependent inhibition of cell growth was observed in VSMCs incubated with calpeptin, a cell-permeable calpain inhibitor that is potent against both μ- and m-calpain.33 This inhibitory effect of calpeptin was not due to the toxicity of this compound, because VSMCs could grow after the removal of calpeptin in culture medium (data not shown). Comparable growth inhibition with calpeptin was also observed in VSMCs treated with benzylxycarbonyl-Leu-Met-H, which is 17 times more potent as an inhibitor against m-calpain than against μ-calpain.33 The serine protease inhibitor leupeptin, which is less permeable and less specific for calpain, also showed mild but significant inhibition of VSMC growth. Antisense oligonucleotide against m-calpain also inhibited VSMC growth in a dose-related manner, whereas control oligonucleotide showed no inhibitory effect on VSMC growth.

**DNA Flow Cytometry**

We then carried out DNA flow cytometric studies at the G1/S interface 24 hours after the addition of antisense oligonucleotide against m-calpain or calpeptin. As shown in Fig 5, the admixture of antisense oligonucleotide against m-calpain or calpeptin, but not control oligonucleotides, leads to a 50% reduction in the numbers of cells entering the S phase.

**Discussion**

It has been well established through the direct examination of 

\[ [Ca^{2+}] \]

in the cell cycle that 

\[ [Ca^{2+}] \]

transients are involved in cell growth.1-10 Although several studies have also suggested possible mechanisms to increase the 

\[ [Ca^{2+}] \]

integral to cell proliferation,2,3,8-10 it is still controversial how 

\[ [Ca^{2+}] \]

transients contribute to cell cycle progression.8-10 In addition to 

\[ [Ca^{2+}] \],
transients, a possible role of protease in cell growth has been suggested by several studies using protease inhibitors; however, those studies failed to specify the protease responsible for cell growth, partly because of a lack of specific inhibitors. Through this knowledge on the role of \([\text{Ca}^{2+}]\), transients and protease in cell growth, one can easily hypothesize that calpain, a calcium-activated neutral protease, may possess important roles as an effector of \([\text{Ca}^{2+}]\), rise in cell growth, and this hypothesis has been strengthened by the recent findings of important transcription factors as calpain substrate, such as nuclear factor-\(\kappa\)B, \(c-fos, ffa-2, fos-b, jun-b, c-jun,\) and \(jun-d.35,36\) However, several attempts have failed to clarify the exact roles of calpain in cell growth. Schollmeyer\(^2\) microinjected m-calpain into PtK1 cells to observe progression in mitosis, whereas March et al.\(^{26}\) used several protease inhibitors to inhibit cell growth at the \(G_1/S\) interface. This discrepancy has not been explained. Although some of the inhibitors could abolish the activity of calpain,

![Figure 3](image3.png)

**Figure 3.** Western blot analysis of m-calpain, calpastatin, or pp125FAK in VSMCs treated with calpeptin and m-calpain antisense oligonucleotide. VSMCs growth-arrested by incubation in 0.5% FBS-DMEM (growth-arrest medium) for 96 hours were then incubated in 10% FBS-DMEM containing control saline, calpeptin, its analogue (BOC-Leu-Met-H), scramble antisense oligonucleotide, or m-calpain antisense (As) oligonucleotide. Cells were solubilized in SDS sample buffer and subjected to Western blot analysis as described in “Methods.” The contents of m-calpain were densitometrically examined, and the content of m-calpain in VSMCs treated with control saline was considered to be 100% (A). Data represent mean ± SD. \(n=5; \quad * P<.05.\) Results shown are from one representative of four different experiments.

![Figure 4](image4.png)

**Figure 4.** Effects of protease inhibitors and m-calpain antisense oligonucleotide on VSMC growth. VSMCs growth-arrested by incubation in 0.5% FBS-DMEM (growth-arrest medium) for 96 hours were then incubated in 10% FBS-DMEM containing control saline, calpeptin, its analogue (BOC-Leu-Met-H), scramble antisense oligonucleotide, or m-calpain antisense (As) oligonucleotide. The cells were permitted to grow for 72 hours and then were trypsinized and counted on a Coulter counter. Data represent mean ± SD. \(n=5; \quad * P<.05.\) Results shown are from one representative of four different experiments.

![Figure 5](image5.png)

**Figure 5.** Effect of m-calpain antisense (As) and calpeptin on cell-cycle progression in VSMCs. VSMCs growth-arrested by incubation in 0.5% FBS-DMEM (growth-arrest medium) for 96 hours were then incubated in 10% FBS-DMEM containing control saline, calpeptin, scramble antisense oligonucleotide, or m-calpain antisense oligonucleotide. VSMCs were loaded with Hoescht 33342 by addition of 4 mL of 10 mmol/L solution of dye to 4 mL of cell culture and incubation of the cells for 1 hour at 37°C. The cells were analyzed by FACStar flow cytometer after trypsinization. Results shown are from one representative of four different experiments.

![Figure 6](image6.png)

**Figure 6.** Western blot analysis of m-calpain, calpastatin, or pp125FAK in VSMCs treated with calpeptin and m-calpain antisense oligonucleotide. VSMCs growth-arrested by incubation in 0.5% FBS-DMEM (growth-arrest medium) for 96 hours were then incubated in 10% FBS-DMEM containing control saline, calpeptin, scramble antisense oligonucleotide, or m-calpain antisense (As) oligonucleotide. Cells were solubilized in SDS sample buffer and subjected to Western blot analysis as described in “Methods.” The contents of m-calpain were densitometrically examined, and the content of m-calpain in VSMCs treated with control saline was considered to be 100% (A). Data represent mean ± SD. \(n=5; \quad * P<.05.\) Results shown are from one representative of four different experiments.
none were specific for calpains. Zhang et al. used a new strategy of antisense oligonucleotide against calpain, which inhibited cell growth, but their results were insufficient because they used antisense oligonucleotide against a 30-kD small subunit of calpain. Although their approach may have the benefit of affecting both \( \mu \)- and m-calpain molecules, Meyer et al. recently reported that an 80-kD large subunit of calpain expressed without a 30-kD small subunit showed proteolytic activity, suggesting that modification of the 80-kD large subunit responsible for proteolytic activity is essential to clarify the exact roles of calpains. In this study, in conjunction with specific calpain inhibitors with unique inhibitory spectra, we used antisense oligonucleotide against m-calpain because our results showed, by means of immunoreactivity, that the predominant isozyme of calpain in VSMCs is m-calpain, and we could successfully present the results confirming the involvement of m-calpain in VSMC growth.

Consistent with previous studies using protease inhibitors, the cell-permeable calpain inhibitor calpeptin inhibited cell growth at the G1/S interface, and the comparable inhibitory effect was attained by the analogue of calpeptin, which is 17 times more potent against m-calpain than against \( \mu \)-calpain. Although a mild inhibitory effect of leupeptin on VSMC growth might suggest a possible contribution of extracellular protease activities on cellular functions, it is impossible to speculate on the exact inhibitory mode of leupeptin, because the possibility exists that the cell-impermeable inhibitor leupeptin was incorporated into the cytosol after prolonged incubation at high extracellular concentrations. Thus, in this study, we focused on intracellular proteases. These observations strongly suggested the involvement of m-calpain, not \( \mu \)-calpain, in VSMC growth; however, these inhibitors still have cross-inhibitory reactivity against other cytoplasmic proteases, such as cathepsin B, H, and L. Thus, we used antisense strategy to confirm the role of m-calpain in VSMC growth.

References

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