Inhibitory effects of ameloblastin on epithelial cell proliferation

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Running title: Characterization of ameloblastin protein

Abstracts

Objective: Ameloblastin is an enamel matrix protein expressed in several tissues. Many potential mechanisms have been identified by which ameloblastin functions as an extracellular matrix protein. However, the biological effects of ameloblastin on gingival epithelial cells remain unclear. In the present study, we established a novel system to purify recombinant human ameloblastin and clarified its biological functions in epithelial cells *in vitro*.

Design: Recombinant human ameloblastin was isolated from COS-7 cells overexpressing HaloTag®-fused human ameloblastin by the HaloTag® system and then purified further by reverse-phase high-performance liquid chromatography. SCC-25 cells, derived from human oral squamous cell carcinoma, were treated with recombinant ameloblastin and then cell survival was assessed by a WST-1 assay. Cell cycle analysis was performed by flow cytometry.

Results: The novel purification system allowed effective recovery of the recombinant ameloblastin proteins at a high purity. Recombinant ameloblastin protein was found to suppress the proliferation of SCC-25 cells. Flow cytometric analysis showed that ameloblastin treatment induced cell cycle arrest G1 phase.

Conclusions: We developed a procedure for production of highly purified recombinant human ameloblastin. Biological analyses suggest that ameloblastin

induces cell cycle arrest in epithelial cells and regulates the progression of periodontitis.

Keywords:

Ameloblastin

Protein purification

Periodontitis

Epithelial cell proliferation

Cell cycle

Apoptosis

1. Introduction

Periodontitis is one of the most common chronic inflammatory diseases characterized by the destruction of tooth-supporting structures.[1] It is a complex disease resulting from the combination of the direct effects of microbial virulence factors and the host response to microbial challenge.[2,3] Although numerous microbial etiologies have been reported for periodontitis, its pathogenesis remains to be elucidated because of the complexity of host-microbial interactions.

The gingiva is covered by a stratified squamous epithelium that constantly receives local stimuli. The gingival epithelium can serve as the first line of defense against bacterial invasion.[4] In addition to its function as a physical and

chemical barrier, the gingival epithelium plays a crucial role in the immune response against infectious inflammation in periodontal tissue by expression of a large variety of cytokines and antimicrobial peptides.[5,6,7,8] Therefore, the gingival epithelium plays a critical role in maintaining mucosal homeostasis, and the loss of the epithelial barrier function is thought to contribute towards periodontitis progression.

Ameloblastin, a matrix adhesion protein also known as sheathlin and amelin, was first detected in secretory stage ameloblasts.[9,10] It is also expressed by osteoblasts,[11] cementoblasts,[12] and epithelial rests of Malassez in the periodontal ligament.[13] Ameloblastin contains a fibronectin interaction sites,[14] several heparin-binding domains,[15,16] a potential α2β1 integrin-binding domain, and a thrombospondin cell adhesion motif,[17] which might be responsible for the interaction of ameloblastin with the surface of dental epithelial cells.

Ameloblastin was initially reported to involved in the regulation of ameloblasts.[18] Many potential mechanisms have been identified by which ameloblastin functions as an extracellular matrix protein, including the regulation of enamel biomineralization[19,20,21] and osteoblast differentiation.[22] Furthermore, recent studies have suggested that ameloblastin may act as a signaling molecule[23, 24] and possesses growth factor activity.[25]

In our previous study, we purified bioactive fractions from enamel matrix derivative (EMD) and revealed that ameloblastin is a candidate component that inhibitas epithelial cell proliferation (unpublished data). However, the detailed

mechanism by which ameloblastin inhibits epithelial cell proliferation has not because of the difficulty of discriminating the responses induced by ameloblastin from those caused by other proteins that often contaminate ameloblastin preparations. In the present study, we purified recombinant ameloblastin protein using a novel fusion tag system and clarified its biological functions in epithelial cells *in vitro*.

2. Materials and methods

2.1. *Cell culture*

SCC-25 cells, derived from human squamous cell carcinoma of the tongue, were obtained from DS Pharmaceutical (Osaka, Japan) and maintained in a 1:1 mix of Dulbecco's modified Eagle's medium and Ham's F-12 (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), penicillin G (100 U/ml) and streptomycin (100 μg/ml).

2.2. *Expression and purification of recombinant ameloblastin*

The expression vector pFNA21A (FHC21950M; Promega KK, Tokyo, Japan) was used to express HaloTag®-fused human ameloblastin protein. Expression plasmids were transfected into COS-7 cells by electroporation using a NEPA21 Super Electroporator (Nepa Gene, Chiba, Japan). After 24 hours, the transfected cells were lysed using a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton

X-100, and 0.1% sodium deoxycholate, pH 7.5). HaloTag® fusion recombinant protein was then partially purified by the HaloTag® Mammalian Protein Purification System (Promega KK) according to the manufacturer's instructions. The isolated fraction was lyophilized and then the recombinant ameloblastin was further purified by reverse-phase high-performance liquid chromatography (HPLC) using a Waters system (Medford, MA, USA) and C_{18} column (4.6 × 150 mm; Vydac, Hesperia, CA, USA) equilibrated with 0.1% trifluoroacetic acid. The fraction with one major peak was collected, diluted in culture medium, sterilized using a surfactant cellulose acetate membrane filter (0.20 μm pore size; Corning, NY, USA), and then used as purified recombinant ameloblastin in bioassays. Protein concentrations were measured using a DC ™ protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

2.3. *Preparation of a monoclonal antibody (mAb) against human ameloblastin*

Animal immunization was performed by a standard procedure using recombinant ameloblastin as the immunogen. Ameloblastin (1.0 ml, 15 μg) was emulsified in an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI, USA) and then injected intraperitoneally into 7-week-old female Balb/c mice (Charles River Laboratories Japan, Yokohama, Japan). Then, at about 2-week intervals, Balb/c mice were administered with a booster injection of ameloblastin prepared in the same manner. After the second immunization, the titer of antiserum was tested by an enzyme linked immunosorbent assay (ELISA).

Recombinant ameloblastin was diluted in coating buffer (50 mM $NaHCO_3$) to 1 μg/ml, added to the micro-titer plates (50 μl/well) as the coating antigen, and then incubated overnight at 4℃. Plates coated with recombinant ameloblastin were washed three times with PBST (PBS containing 0.05% Tween-20) and then blocked with 1% Block Ace (DS Pharma Biomedical, Osaka, Japan) at room temperature for 2 hours. After three washes with PBST, serial dilutions of antiserum (50 μl/well) were added to the plates, followed by jncubation at room temperature for 2 hours. After washing, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBST was added to the reaction wells, followed by jucubation at room temperature for 30 minutes. After five washes with PBST, SigmaFast OPD tablet set (Sigma-Aldrich, St Louis, MO, USA) was added to the wells for color development, and then 10% H_2SO_4 was added to stop the reaction. The absorbance was measured at 490 nm by a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

The immunized mouse with a high serum titer was given an intravenous injection with of 5 μg ameloblastin. After 4 days, the B cells were isolated from spleen and fused with P3U1 myeloma cells at a ratio of 1:5 by polyethylene glycol 1500. Then, the cells were cultured in 96-well plates containing HAT medium at 37℃ with 5% CO_2. After about 10 days, the HAT medium was changed to HT medium. After 2 days, the culture supernatants were tested by ELISA and positive clones were obtained by limiting dilutions until the positive percentage

reached 100%. Preparation of the mAb from hybridoma supernatants was carried out using a HiTRAP Protein G HP affinity column (GE Healthcare UK, Amersham, Buckinghamshire, UK). The reactivity and specificity of the purified mAb were analyzed by western blotting.

2.4. *Western blotting*

Purified ameloblastin protein was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and the separated proteins were transferred to a polyvinylidene fluoride membrane (Immobilon P; Millipore, Billerica, MA, USA). Nonspecific binding sites were blocked by incubating the membrane in Blocking One (Nacalai Tesque) at room temperature for 60 minutes. The membrane was then incubated with an anti-ameloblastin polyclonal antibody (Santa Cruz Biotechnology) or the anti-ameloblastin mAb at 4°C overnight. Immune complexes were detected by incubation with an HRP-conjugated secondary antibody (Molecular Probes, Carlsbad, CA, USA) at room temperature for 1 hour. After washing the membranes, chemiluminescence was produced using ECL reagent (Amersham Pharmacia Biotech, Uppsala, Sweden) and detected digitally with the GelDoc ^{TM} XR Plus System (Bio-Rad Laboratories).

2.5. *WST-1 analysis*

Cell viability was determined using tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2H-5-tetrazolio]-1-3-benzene disulfonate; Dojindo

Laboratories, Kumamoto, Japan). SCC-25 cells (1×10^4 cells/well) were seeded in 96-well plates and cultured for 6 hours. The cells were then stimulated with recombinant ameloblastin for 48 hours. WST-1 solution (10 µl) was then added to each well, followed by incubation for 2 hours. Absorbances at 450 and 630 nm were measured using a Multiskan JX Microplate Reader (Thermo Electron, Kanagawa, Japan).

2.6. *Flow cytometric analysis*

To analyze the cell cycle, SCC-25 cells (1.5×10^5 cells/ml) were suspended in a hypotonic solution (0.1% sodium citrate, 0.2% NP-40, and 0.25 mg/ml RNase, pH 8.0) and then stained with 50 µg/ml propidium iodide (PI). DNA content was then analyzed using an EPICS XL (Beckman Coulter, Fullerton, CA, USA). The percentage of cells in each cell cycle phase was determined by MultiCycle for Windows (Phoenix Flow Systems, San Diego, CA, USA).

2.7. *Statistics analysis*

Data are expressed as mean values ± standard deviation (SD) of three experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by Dunnett's test. A value of $P < 0.05$ was considered significant. All statistical analyses were carried out using JMP® software version 10.0.2 (SAS Institute, Cary, NC, USA).

3. Results

3.1. *Purification of recombinant human ameloblastin*

Halo-ameloblastin and cleaved ameloblastin were purified using the HaloTag® Mammalian Protein Purification System (Fig. 1). First, the Halo-ameloblastin fusion protein was subjected to HaloLink ™ Resin. This step removed most of the other proteins. Purified Halo-ameloblastin was then digested by HaloTEV Protease. After dialysis, the cleaved ameloblastin protein was separated from residual uncleaved Halo-ameloblastin by reverse-phase HPLC.

Western blot analysis of recombinant ameloblastin showed a 105 kDa Halo-ameloblastin fusion protein before cleavage (Fig. 2A, 2B, lane 1). After cleavage, we detected a predominant immunoreactive band of 70 kDa (Fig. 2A, 2B, lane 5). As shown in Figure 2C, ameloblastin was only observed in the fraction with a high peak in reverse-phase HPLC purification, whereas other fractions showed no immunoreactive band.

3.2. *Effects of purified recombinant ameloblastin on epithelial cell proliferation*

To determine the effects of ameloblastin on cell viability, SCC-25 cells were treated with purified ameloblastin for 48 hours, and then cell survival was assessed by a WST-1 assay. The proliferation of SCC-25 cells was inhibited when cultured with recombinant ameloblastin (Fig. 3). The effect of ameloblastin

on cell proliferation was concentration-dependent and reached a maximum at 25 ng/ml (88% inhibition). Cells treated with the vehicle (1 × PBS, pH 7.5, containing 1 mM dithiothreitol and 0.005% IGEPAL® CA630) showed no effects on cell viability (data not shown).

3.3. *Cell cycle arrest in epithelial cells induced by ameloblastin*

The effect of ameloblastin on the cell cycle of SCC-25 cells was examined by flow cytometric analysis. No change in the percentage of control cells treated with the vehicle in each phase was observed during the culture period (Fig. 4A). In contrast, the percentage of ameloblastin-treated cells in the G1 phase increased from 55.2 to 69.3% over 48 hours of culture (Fig. 4B).

4. Discussion

Although cultured mammalian cells are preferred to produce functional mammalian proteins with appropriate post-translational modifications, purification of recombinant proteins is frequently hampered by low expression. The development of fusion tag systems is essential for purification and analysis of recombinant proteins.[26] The advantages of using fusion proteins to facilitate purification and detection of recombinant proteins are well recognized because of their reversible binding interactions with specific ligands. HaloTag® is a 34 kDa monomeric protein derived from a bacterial haloalkane dehalogenase, which was

designed to covalently bind to a series of chloroalkane ligands.[27] Covalent bond formation between the protein tag and the chloroalkane linker is highly specific and occurs rapidly under physiological conditions.[28] Furthermore, cleavage of the HaloTag® does not result in protein insolubility. In the present study, we purified recombinant human ameloblastin from COS-7 cells overexpressing HaloTag®-fused human ameloblastin using the HaloTag® Mammalian Protein Purification System, followed by further purification using reverse-phase HPLC (Fig. 1). As shown in Figure 1B and 1C, our purification system was found to be effective to purity and recover the recombinant ameloblastin proteins.

Western blotting analysis revealed that this novel method allowed highly efficient purification of recombinant ameloblastin (Fig. 2A). We also generated a highly specific mAb against human ameloblastin. In general, mAbs are used in scientific studies because of their specific and high affinity interactions with antigens. As shown in Figure 2B and 2C, the anti-ameloblastin mAb exhibited high sensitivity and specificity for recombinant ameloblastin. These findings suggest that the mAb established in this study may facilitate further studies of the structure and function of ameloblastin and permit large-scale production of biologically active recombinant ameloblastin that is suitable for *in vivo* experiments.

Amelogenin and ameloblastin have been reported to exhibit growth factor-like activities in periodontal ligament cells.[25] However, less attention has been paid to the effects of ameloblastin on epithelial cell proliferation. In the

present study, recombinant ameloblastin exhibited an inhibitory effect on epithelial cell proliferation *in vitro* (Fig. 3). Our results suggest that ameloblastin suppresses initial periodontal destruction by inhibiting epithelial cell proliferation. Conversely, a previous study has demonstrated that ameloblastin stimulates the proliferation of human periodontal ligament cells.[25] These findings indicate that ameloblastin has differential effects on cell proliferation according to the cell type. We have no precise explanation for this phenomenon, but we speculate that the difference in viability may be because of receptor expression levels. Ameloblastin has been shown to interact with CD63,[22] although the physiological roles of this interaction remain unclear. The molecular mechanisms for the interaction between ameloblastin and CD63 are currently under investigation using mAbs established in our laboratory.

Cell growth is thought to be critically regulated by the cell cycle.[29,30] We found that ameloblastin treatment inhibited cell cycle arrest at the G1 phase (Fig. 4). The oral epithelium prevents invasion by pathogenic microorganisms and is important for periodontal wound healing. However, the down growth of epithelial cells occurs in periodontium affected by periodontitis. The presence of ameloblastin in the gingival sulcus may contribute to the prevention of down growth of the epithelium in the initiation of periodontitis. These findings show provide a valuable insight into how ameloblastin may alter epithelial turnover in the prevention of periodontal breakdown.

Acknowledgements

This study was partially supported by a Grant-in-Aid for subject-solution-type development of a medical instrument from the Ministry of Economy, Trade and Industry of the Japanese government.

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Figure legends

Fig. 1. Purification of recombinant ameloblastin protein

Scheme of the protocol for recombinant ameloblastin purification.

Fig. 2. Detection of recombinant ameloblastin protein

Purified ameloblastin was subjected to 12.5% SDS-PAGE and western blot analyses. Blotted membranes were treated with a polyclonal antibody (A) and mAb (B) against ameloblastin. Lanes: 1, Total cell lysate transfected with the

expression vector for HaloTag®-fused human ameloblastin proteins; 2, Flow-through after the sample was loaded onto HaloLink™ Resin; 3, HaloTag® Protein Purification Buffer wash; 4, Sample taken after inducing cleavage with TEV protease; 5, Final protein purified by reverse-phase HPLC. (C) Each fraction purified from reverse-phase HPLC was also subjected to 12.5% SDS-PAGE and western blot analysis using the anti-ameloblastin mAb.

Fig. 3. Effect of recombinant ameloblastin on epithelial cell proliferation

SCC-25 cells were stimulated with recombinant ameloblastin for 48 hours, and then cell viability was determined by WST-1 analysis. Data show the percentage inhibition of cell proliferation from three independent samples. Bars represent the means + SD. Data were analyzed by Dunnett's test after one-way ANOVA (***$P <$ 0.0001 compared with the vehicle alone).

Fig. 4. Effect of ameloblastin on the cell cycle in epithelial cells

SCC-25 cells were treated with ameloblastin (100 ng/ml) for the indicated times, and then stained with PI to determine the distribution during each phase of the cell cycle. (A) Vehicle-treated control cells. (B) Ameloblastin-treated cells. Data shown are representative of three independent experiments with similar results obtained in each. Solid bars percentage of cells in G1 phase; open bars percentage of cells in S phase; striped bars presentage in G2/M phase.

Figure 1
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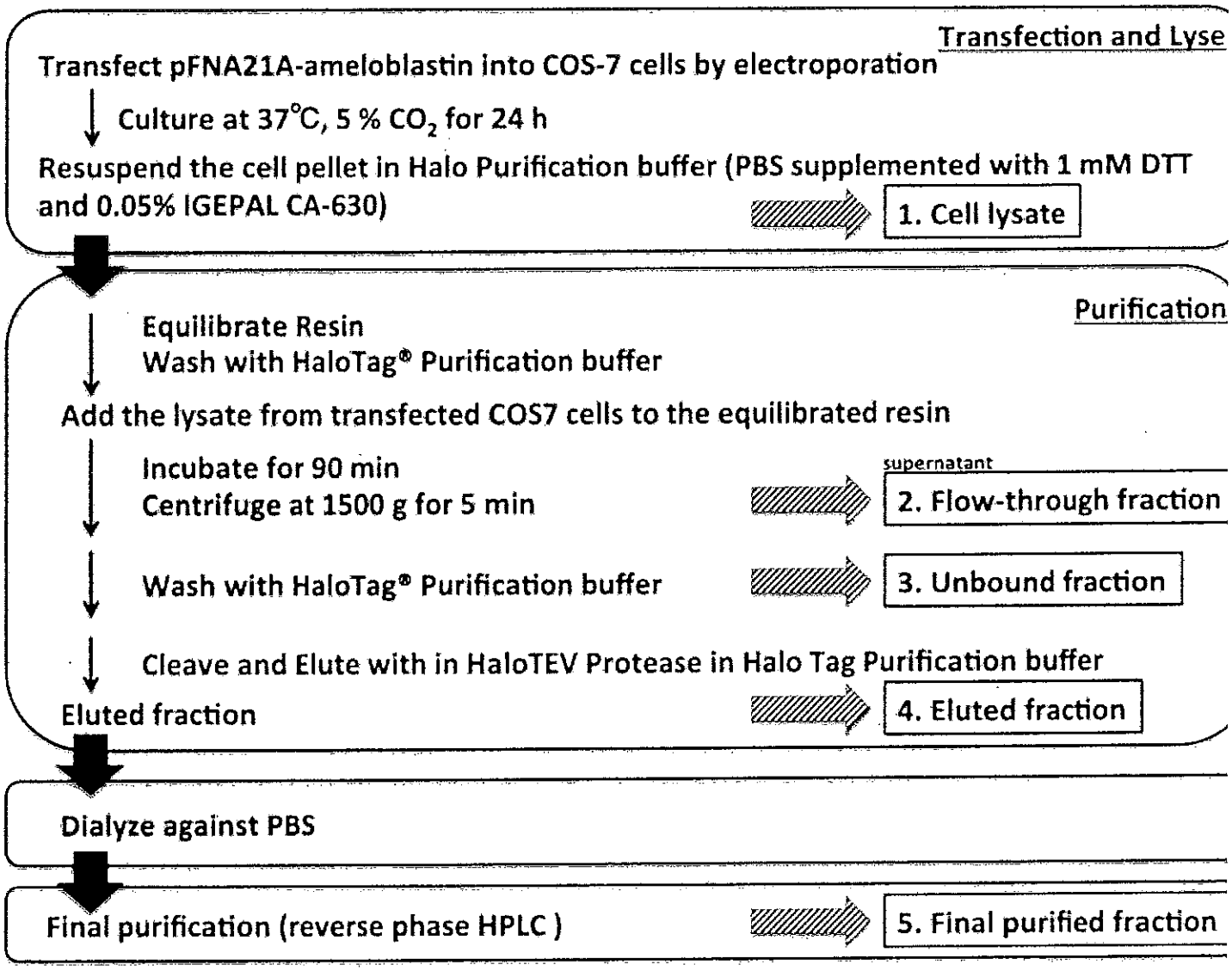

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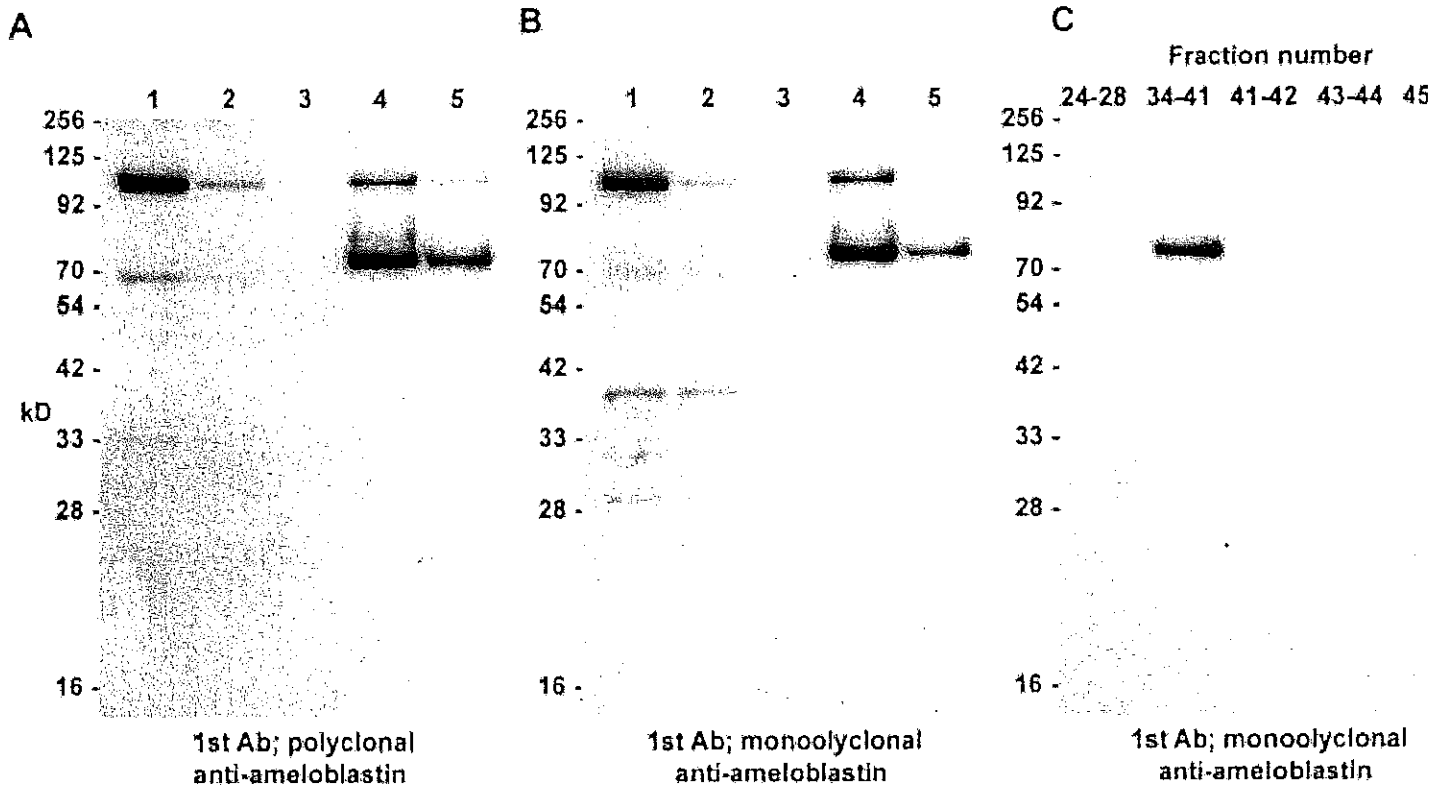

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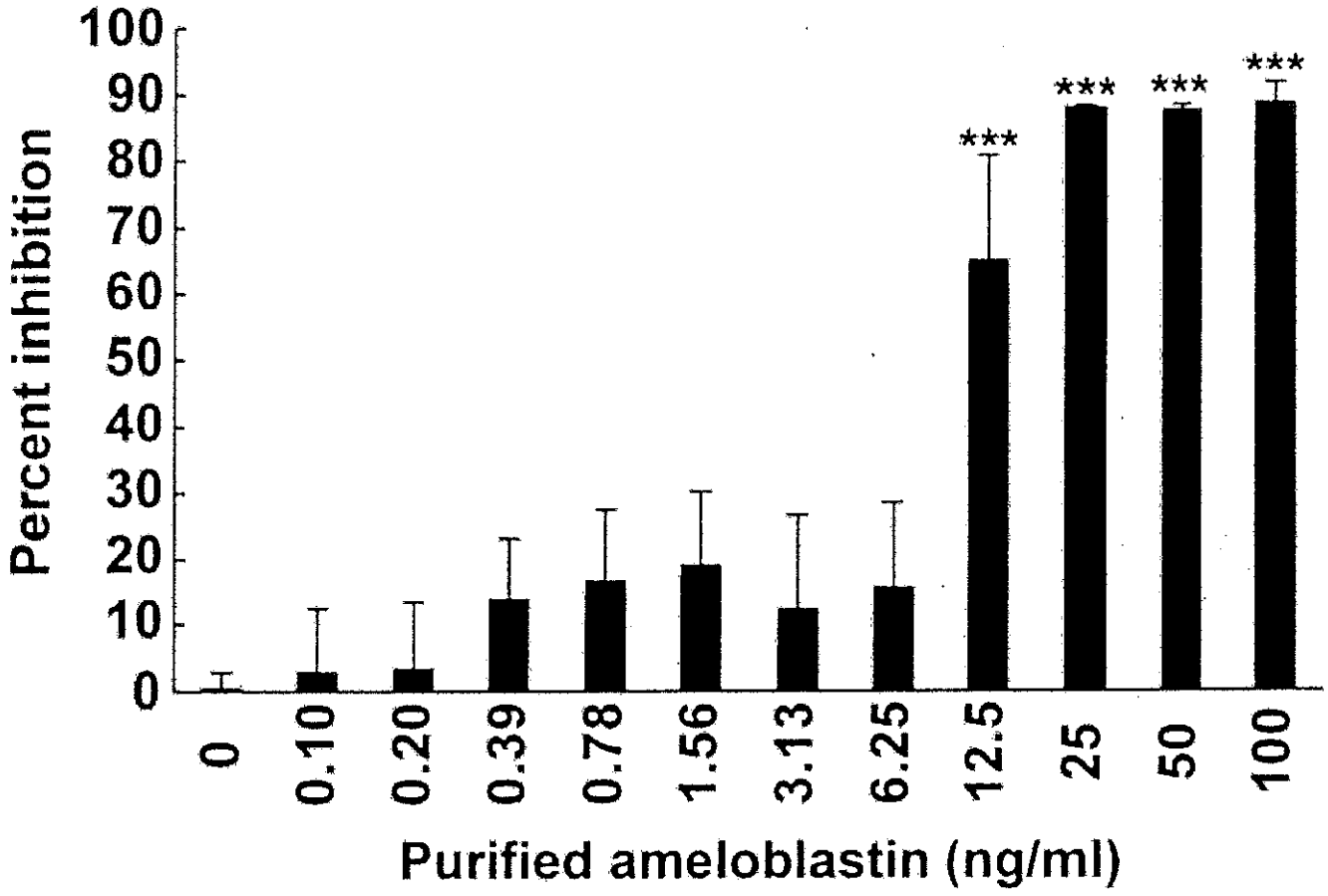

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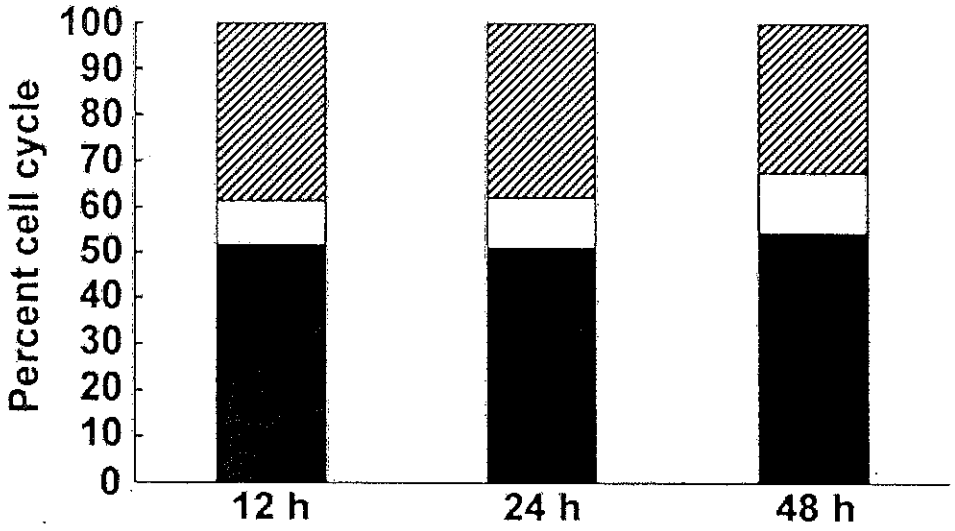
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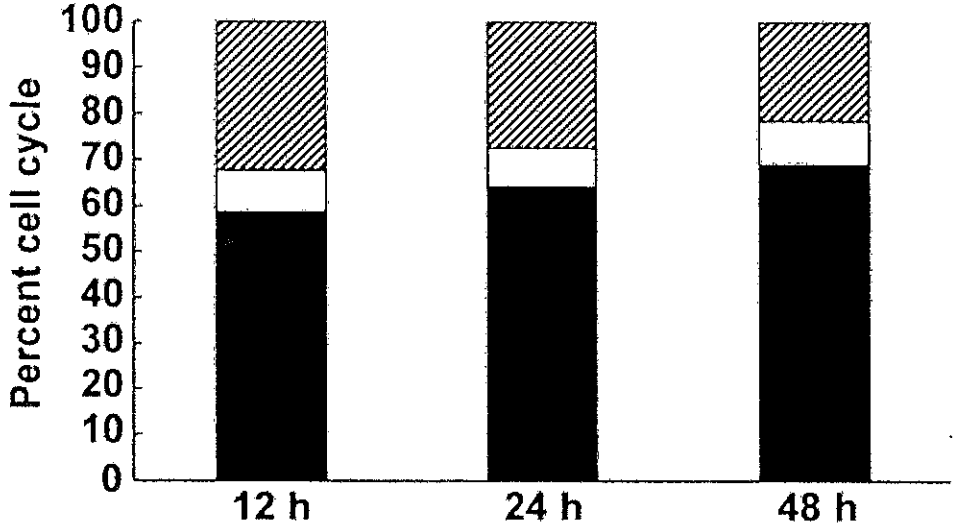
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