PROTEASES POTENTIATE FIBROCYTE DIFFERENTIATION

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Proteases Potentiate Fibrocyte Differentiation. (May 2014)

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45% of deaths in the United States involve fibrosing diseases and the treatment of non-healing chronic wounds costs more than $25 billion annually in the United States alone. Scar tissue formation plays a critical role in both wound healing, and fibrosis. Currently, there is not a solid understanding of the mechanism that triggers initiation of scar tissue formation in healing wounds and fibrosing diseases such as chronic kidney disease, scleroderma, chronic asthma, and idiopathic pulmonary fibrosis. Fibrocytes are fibroblast-like cells involved in the formation of scar tissue in healing wounds and fibrosing disease. The time required to form scar tissue in a wound is related to the amount of fibrocytes present. As fibrocyte differentiation increases, scar tissue forms faster; however, if fibrocyte differentiation is hindered, then scar tissue takes longer to form. Thrombin and tryptase are proteases involved in both wound healing and fibrotic lesions, while trypsin has been used topically to speed wound healing. Adding trypsin, thrombin and tryptase to PBMC isolated from donor's blood increases the amount of fibrocyte differentiation, suggesting that these proteases play a critical role in the potentiation of fibrocyte differentiation.

In addition to potentiating fibrocyte differentiation, these proteases also increase the proportion of M2a pro-fibrotic macrophages as well as collagen production, both makers for scar tissue formation. Fibrocyte differentiation is greatly inhibited by the presence of the plasma protein Serum Amyloid P (SAP). Tryptase and thrombin potentiate fibrocyte differentiation in the presence of SAP, suggesting that these proteases can be part of a triggering mechanism for scar tissue formation in both healing wounds and fibrotic lesions. Consequently, proteases may be an effective treatment for non-healing wounds, and protease inhibitors could be an effective treatment for fibrosing diseases.

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CHAPTER I

INTRODUCTION

Fibrosing diseases are involved in 45% of the death in the United States [1]. The more than 62 known fibrosing diseases affect major organs such as the heart, liver and kidneys, as well as tissue [1]. Also, other fibroprofliferative fibrosing disorders include scleroderma, keloids, and atherosclerosis [1]. Fibrocytes are involved in both wound healing and scar tissue formation, and are found in a variety of disease settings such as chronic asthma, idiopathic pulmonary fibrosis, nephrogenic systemic pulmonary fibrosis, and scleroderma; therefore, inhibiting fibrocyte differentiation in these disorders could be used as a therapeutic.

Chronic wounds affect more than 6.5 million patients and cost $25 billion per year to treat in the United States alone. Non-healing chronic wounds predominately affect elderly, obese, and diabetic patients [2]. Wound healing is an active process, which involves blood cells, soluble intermediates, extracellular matrix, prenchymal cells [3]**,** angiogenesis, and interactions between epidermal and dermal cells; these complex interactions are controlled by different cytokines and growth factors [4]**.** Successful wound healing can be divided in three main phases: inflammation, proliferation, and remodeling. During the inflammatory phase neutrophils predominate, which allows the wound site to be as clean as possible by removing bacteria and other foreign substances found at the wound site. Next, during the proliferation phase fibroblasts play a critical role, because they produce extracellular matrix proteins, collagen and fibronectin; thus, fibroblasts are fundamental for scar tissue formation. It is during this phase that fibrocytes participate in wound healing; monocytes (circulating blood leukocytes) [5], are recruited to

injured tissue by different chemokines. Monocytes differentiate into fibrocytes, which are fibroblast- like cells that are express CD45+, and secrete collagen, which allows them to form scar tissue and contribute to the wound healing process [6]. The rate at which a wound heals is related to the amount of fibrocytes present at the wound site; that is, increasing the amount of fibrocytes present within a wound potentiates wound healing, while decreasing the amount of fibrocytes present within a wound hinders wound healing [2]**.** The final phase of wound healing is remodeling, which is a process that occurs after the wound has closed [4].

Trypsin, tryptase, and thrombin are proteolytic enzymes that have different roles in the body. Previous data indicates that proteolytic enzymes can increase the rate at which a wound heals [7]. Also, trypsin can speed up wound healing; thus, trypsin has been used in the past in clinical application related to wound healing. Trypsin is especially helpful since it can remove any debris present at the wound site, decrease edema, and act as an anticoagulant, all these characteristics allow trypsin to speed the rate at which a wound heals [8]. Tryptase also plays a role in wound healing. Tryptase is secreted by mast cells in wounds and fibrotic lesions and causes fibroblasts to proliferate and secrete collagen [9]. Thrombin is directly involved in the wound healing process as the main protease of the coagulation cascade. Thrombin plays a role in the formation of the fibrous matrix of blood clots; this is accomplished by converting fibrinogen to fibrin. Also, thrombin contributes to platelet formation by triggering shape changes in platelets and releasing platelet activating factors [10]. This report shows that proteolytic proteins such as trypsin, thrombin and tryptase potentiate fibrocyte differentiation, which offers a feasible solution to the problem of wound healing.

It has been previously shown that trypsin requires protein supplements (albumin, insulin, and transferrin) in order to potentiate fibrocyte differentiation [2]. This suggests that trypsin acts on protein supplements, or that protein supplements are necessary in order for trypsin to potentiate fibrocyte differentiation [2]. We tested the ability of trypsin, thrombin, and tryptase to potentiate fibrocyte differentiation on different supplement-containing media.

CHAPTER II

METHODS

PBMC isolation and culture

Human blood was collected from adult volunteers after giving written consent and with specific approval from the Texas A&M University human subjects Institutional Review Board. Peripheral blood mononuclear cells (PBMC) were isolated and cultured as previously described [12,3]. Protein-free media (PFM) was Fibrolife basal media (Lifeline Cell Technology, Walkersville, MD) supplemented with 10 mM HEPES (Sigma, St. Louis, MO), 1× non-essential amino acids (Sigma), 2 mM glutamine (Lonza, Basel, Switzerland), 1 mM sodium pyruvate (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (Lonza). Serum-free media (SFM) was PFM containing 10 μg/ml recombinant human insulin (Sigma), 5 μg/ml recombinant human transferrin (Sigma), and 550 μg/ml filter sterilized human albumin (purified as previously described [2]), casein from bovine milk (Sigma), ovalbumin from egg white (Acros Organics, Waltham, MA), skim milk powder (EMD Millipore), bovine skin gelatin (Sigma), or fish skin gelatin (Sigma). For casein the pH was raised to 9, to get the 5 mg/ml protein into solution. The pH was brought back to 8 prior to addition to PBMC. Supplements were checked for concentration using absorbance at 280 nm. SFM made with each supplement at 500 ug/ml was mixed with trypsin at 500 ng/ml for 24 hours at 37˚C and assayed on 4-20% SDS gels (Bio-Rad, Hercules, California), which were silver stained to check for purity and protein digestion. Where indicated, PFM was supplemented to 2.5% (v/v) with sterile filtered non-blood type specific human serum, tested negative for hepatitis A and B and HIV I and II (Lonza and Gemini Bio-products, West Sacramento, California).

Exposure of PBMC to protease

TPCK-treated bovine trypsin (Sigma) and bovine thrombin (Sigma) were resuspended following the manufacturer's instructions. Tryptase purified from human mast cells (Fitzgerald) was mixed with 15 kDa heparin from porcine stomach (Sigma) in a 1:10 molar ratio of tryptase to heparin immediately after thawing [11]. Trypsin, thrombin, and tryptase were added to PBMC in a 96-well plate (BD biosciences) at the indicated concentrations.

Exposure of PBMC to SAP

Human SAP was purified as previously described [12], with the following modifications. Human serum from blood donors was mixed 1:1 with PBS. This serum was mixed with gentle rolling in a 10:1 ratio with SP sepharose beads (GE Healthcare, Piscataway, NJ) in 20 mM Tris, 140 mM NaCl, 2 mM $CaCl_2$ pH 8.0 buffer and eluted as previously described [12]. SAP purification was checked by silver stain on an SDS-PAGE gel and concentration was assessed by absorbance at 280 nm. Proteases were co-incubated with purified SAP to determine if proteases at 500 ng/ml cleaved SAP, which was run on 4-20% SDS gels and silver stained to assess proteolysis of SAP. SAP at 10 ug/ml was co-incubated with PBMC and proteases at the indicated concentration.

Macrophage immunohistochemistry and secreted factors

PBMC were cultured on 8-well tissue-culture treated slides (Millipore) as previously described [13] and exposed to trypsin, tryptase and thrombin at 12.5 ng/ml for five days. PBMC were allowed to dry by air before fixation in acetone for 10 minutes. The cells were stained for CCR7 (mouse monoclonal clone 150503, R & D systems, Minneapolis, MN), CD163 (mouse monoclonal clone GH1/61, Biolegend, San Diego, CA), CD206 (mouse monoclonal clone 15-2,

Biolegend), and fibronectin (rabbit polyclonal, Sigma) as previously described [13]. Conditioned media from PBMC cultured in SFM with or without 12.5 ng/ml protease were analyzed for IL-10 and IL-12 using a human IL-12 ELISA kit (Peprotech, Rocky Hill, NJ) and a human IL-10 ELISA kit (Biolegend), following the manufacturer's instructions.

Gels and staining

4-20% polyacrilamide gels (Bio-Rad) were stained by using silver stain, and using a sequential stains-all stain followed by silver stain where indicated [14]. The concentration of the supplements was 500µg/ml. Proteins were mixed with 5µg/ml trypsin, and left overnight with tumbling at 37ºC.

CHAPTER III

RESULTS

Trypsin, thrombin, and tryptase potentiate fibrocyte differentiation

Fibrocytes are involved in wound healing because they have the ability to secrete collagen and fibronectin. It has been previously shown that trypsin potentiates fibrocytes differentiation, because of this trypsin is commonly used topically in order to speed wound healing. Fibrocytes are fibroblast-like cells involved in the formation of scar tissue in healing wounds. The time required to form scar tissue in a wound is related to the amount of fibrocytes present [15]. Our results show that trypsin potentiates fibrocyte differentiation as expected (Figure 1A); we wanted to determine if other proteases had the same effect on fibrocyte differentiation as trypsin; consequently, we decided to test the effect of tryptase and thrombin on fibrocyte differentiation. Human peripheral blood mononuclear cells (PBMC) were incubated for 5 days with either tryptase or thrombin in order to determine if fibrocyte differentiation was potentiated. After the incubation period the cells were stained, and the number of fibrocytes present under each condition was recorded. PBMC without protease served as a control for the experiment; we observed 388 to 1120 fibrocytes per 10^5 PBMCs from a range of donors; these results agree with previous data [2]. The results indicate that tryptase and thrombin's effects on fibrocyte differentiation are concentration dependent. Tryptase concentrations between 4 and 56 ng/ml and thrombin concentrations between 6 to 12.5 ng/ml increased the number of fibrocytes (Figure 1B-D). Tryptase concentrations above 100 ng/ml and thrombin concentration about 400 ng/ml decreased the number of fibrocytes (Figures 1B and 1C); these results were consistent between different donors. After observing fibrocyte potentiation in the present of tryptase and thrombin,

collagen staining was performed by using flow cytometry. The results show that there is an increase in collagen staining in areas where fibrocyte potentiation occurs (Figure 2A-C). Neither tryptase nor thrombin had a significant effect on the number of adherent cells after the fixing and staining procedures (Figure 3). Consequently, both tryptase and thrombin potentiate fibrocyte differentiation, rather than increasing the cell adhesion of fibrocytes.

Tryptase and thrombin potentiate fibrocyte differentiation in serum-containing media

It has been previously shown that trypsin potentiates fibrocyte differentiation in media containing human serum [2]. We decided to test the ability of tryptase and thrombin to potentiate fibrocyte differentiation in media containing human serum. PBMC were incubated for 5 days in serum containing media in the presence of tryptase or thrombin. Fibrocyte differentiation is inhibited in the presence of serum without tryptase and thrombin (Figure 4); however, potentiation of fibrocyte differentiation occurred in serum containing media having 14 to 446 ng/ml of tryptase and 28 to 112 ng/ml of thrombin. Tryptase and thrombin potentiate fibrocyte differentiation in the presence of human serum at particular concentrations.

Trypsin potentiates fibrocyte differentiation in the absence of albumin

Since trypsin, thrombin, and tryptase potentiate fibrocyte differentiation in the presence of albumin, then it is possible that fibrocyte differentiation is due to the proteases, or to an albumin fragment generated by proteolytic cleavage. To determine if albumin was necessary for the trypsin to potentiate fibrocyte differentiation, we tested the ability of trypsin potentiates fibrocyte differentiation in the absence of albumin, and in the presence of other protein supplements. Six different supplements were tested at 500ug/ml: human albumin, ovalbumin, casein, fish gelatin,

bovine skin gelatin, or skim milk powder. Trypsin potentiated fibrocyte differentiation in media supplemented with fish gelatin, and skim milk powder (Figure 5A and 5B); however, potentiation of fibrocyte differentiation was not observed in media supplemented with casein, ovalbumin, or bovine skin gelatin. SDS-PAGE gels indicated that the protease treatment of protein supplements caused the formation of digestion products in each case (Figures 10A-D). Therefore, albumin is not necessary for potentiation of fibrocyte differentiation to occur in the presence of trypsin.

Trypsin, tryptase, and thrombin potentiate the differentiation of M2a macrophages

Macrophages can differentiate into M1 subsets, which are associated with inflammation, and M2 subsets, which are associated with tissue repair. Additionally, M2a macrophages are a subset of the M2 phenotype. M2a macrophages are associated with scar tissue formation. We wanted to determine if trypsin, thrombin, and tryptase had an effect on M2a macrophage differentiation. PBMC and proteases were co-incubated for five days, and then examined for macrophage markers. CCR7 is a used marker for M1 macrophage activation [16]. Trypsin, thrombin, nor tryptase increased CCR7 staining (Figures 6A and 7A). CD206 are makers of M2 macrophage differentiation used for M2 regulatory and M2a macrophage staining ([16],[17]). Each of the proteases increased CD206 staining, while tryptase and thrombin significantly increased CD163 staining (Figures 6B, 6C, 7B and 7C). Fibronectin is a marker of M2a macrophage differentiation [18]. Our results show that every protease increased fibronectin stating (Figures 6D and 7D). M1 and M2 macrophages differ in their secretion profiles; M1 macrophages secrete higher levels of cytokine IL-12 compared to M2 macrophages [19]. On the other hand, M2 regulatory macrophages secrete increased levels of anti-inflammatory cytokine IL-10 [19].

Trypsin and thrombin decrease M1 macrophage phenotype, for the extracellular accumulation of IL-12 was decreased in the presence of trypsin and thrombin, but not in the presence of tryptase (Figure 8A). On the other hand, tryptase and thrombin decrease the M2 regulatory phenotype, for the extracellular accumulation of IL-10 decreased in the presence of tryptase and thrombin (Figure 8B). Finally, our results indicate that trypsin, thrombin, and tryptase favor macrophage differentiation in PMBC populations towards the M2a phenotype.

Tryptase and thrombin are able to compete with Serum Amyloid P (SAP) to potentiate fibrocyte differentiation

Fibrocyte differentiation is potentiated in the presence of trypsin, tryptase, and thrombin; however, fibrocyte differentiation is inhibited in the presence of Serum Amyloid P (SAP). PMBC were co-incubating with trypsin, thrombin, or tryptase in the presence of 10 ug/ml of SAP, in order to determine if fibrocyte potentiation occurred in the presence of both protease and SAP. 10 ug/ml of SAP completely inhibited fibrocyte differentiation (Figure 9A-D); however, the effect of SAP on fibrocyte differentiation is modified in the presence of tryptase and thrombin (Figure 9B-D), but trypsin did not alter the effect of SAP on fibrocyte differentiation (Figure 9A and 9D). Also, tryptase and thrombin did not cleavage SAP, while trypsin did (Gel not shown).

CHAPTER IV

DISCUSSION

Fibrocytes contribute to the formation of scar tissue in both healing wounds and fibrosing diseases. Chronic wound healing and fibrosing diseases are a major medical issue in today's society. More than 6.5 million people in the United States have a reduced quality of life due to non-healing chronic wounds. Also, the treatment chronic wounds cost more than $25 billion every year in the United States alone. On the other hand, fibrosing diseases account for 45% of deaths in United States [1]. Fibrocyte differentiation is essential to scar tissue formation in both chronic wound healing and fibrosing diseases.

Our data indicates that tryptase and thrombin not only potentiate fibrocyte differentiation, but also collagen production. Fibrocyte potentiation occurred in the presence of protease and fish gelatin or milk powder, indicating that albumin is not necessary for fibrocyte differentiation to occur. Consequently, a protein additive in combination with protease is necessary for proteases to potentiate fibrocyte differentiation.

Tryptase and thrombin effect on fibrocyte differentiation is concentration dependent. Tryptase and thrombin potentiated fibrocyte differentiation between 27 and 112 ng/ml; converting this concentration to a molar value shows that fibrocyte differentiation occurred between 30 to 120 nM. Thrombin peak value was at a concentration of 30nM, which is the same concentration of thrombin in a wound [20].

Scar tissue formation is regulated in the human body by Serum Amyloid P (SAP). Fibrocyte differentiation is inhibited in the presence of SAP [12] , which is a pentameric protein prevalent in serum at a concentration of 30 µg/ml [21]. Our results and previous experiments indicate that SAP inhibits fibrocyte differentiation at a concentration of 10 µg/ml. The question then becomes: how does scar tissue formation begins in the presence of the high SAP concentrations found in serum? Our data indicates a possible explanation to the initiation of scar tissue formation in the body. Both tryptase and thrombin compete with SAP at 10 µg/ml allowing potentiation of fibrocyte differentiation to take place. Both tryptase and thrombin actively participate in wound healing; consequently, our data suggest that tryptase and thrombin allow scar tissue initiation to start at a wound site, due to their ability to compete with SAP.

Macrophages exist in two populations. M1 macrophages participate in inflammation, and M2 participate in remodeling. M1 macrophages upregulate CCR7 and secrete more IL-12, while fibronectin is upregulated in M2a macrophages, which are a subset of M2 macrophages, and are involved in tissue remodeling and scar tissue formation [19]. Our data indicates that trypsin, tryptase and thrombin potentiate both fibrocyte differentiation, and M2a macrophage differentiation. Our results indicate that trypsin, tryptase, and thrombin allow scar tissue formation to take place not only because they potentiate fibrocyte differentiation, but also, because they increase the formation of M2a macrophages.

Millions of people have a reduced quality of life due to the failure of chronic wounds to heal properly. Non-healing chronic wound disproportionately affect at-risk groups, including the elderly, obese and diabetics [2]. Non-healing chronic wounds respond poorly to conventional

treatments [2].There is a need to generate a treatment capable of treating non healing chronic wound. Fibrocytes are involved in scar tissue formation, and as the number of fibrocytes present at a wound site increase, the time required for the wound to heal decreases [15]. Our data shows that trypsin, tryptase, and thrombin potentiate fibrocyte differentiation, while tryptase and thrombin potentiate fibrocyte differentiation in the presence of SAP. Consequently, potentiation of fibrocyte differentiation by proteases at a wound site could be used as a therapeutic for non-healing chronic wounds.

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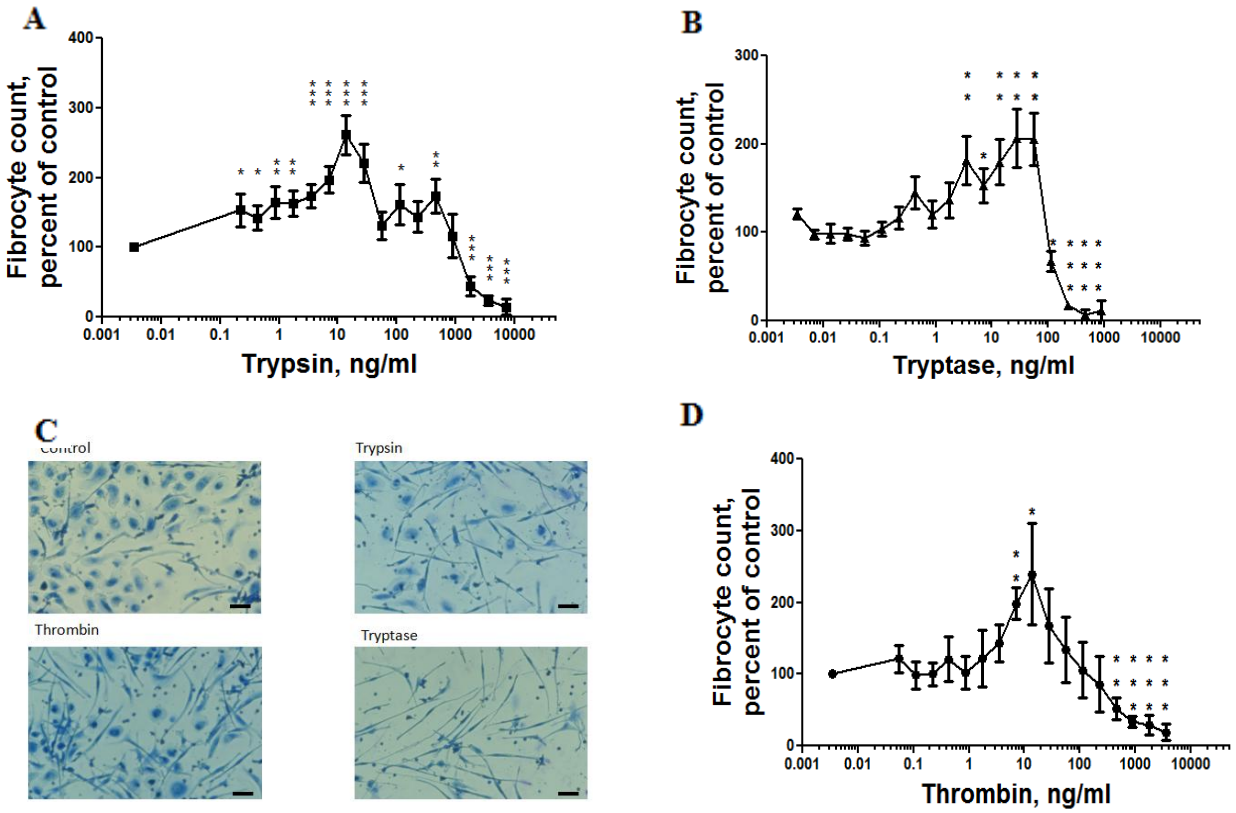

Figure 1. Trypsin, tryptase and thrombin potentiate fibrocyte differentiation. PBMC were cultured in serum free media in the presence of the indicated concentrations of **(A)** trypsin (n=13), **(B)** tryptase (n=10), or **(C)** thrombin (n=7) for 5 days. Fibrocyte counts were normalized for each donor to the no-protease control. The no-protease controls developed 187 ± 22 fibrocytes per 10^5 PBMC. Values in **A** through **C** are mean ± SEM; the absence of error bars indicates that the error was smaller than the plot symbol. * indicates $p < .05$, ** indicates $p < .01$ and *** indicates $p < .001$ compared to the no-protease control by a two tailed t-test. **(D)** Images of PBMC after incubating for 5 days of with no protease (control), 12.5 ng/ ml trypsin, 12.5 ng/ ml thrombin, or 55 ng/ ml tryptase. Bar is 40 µm

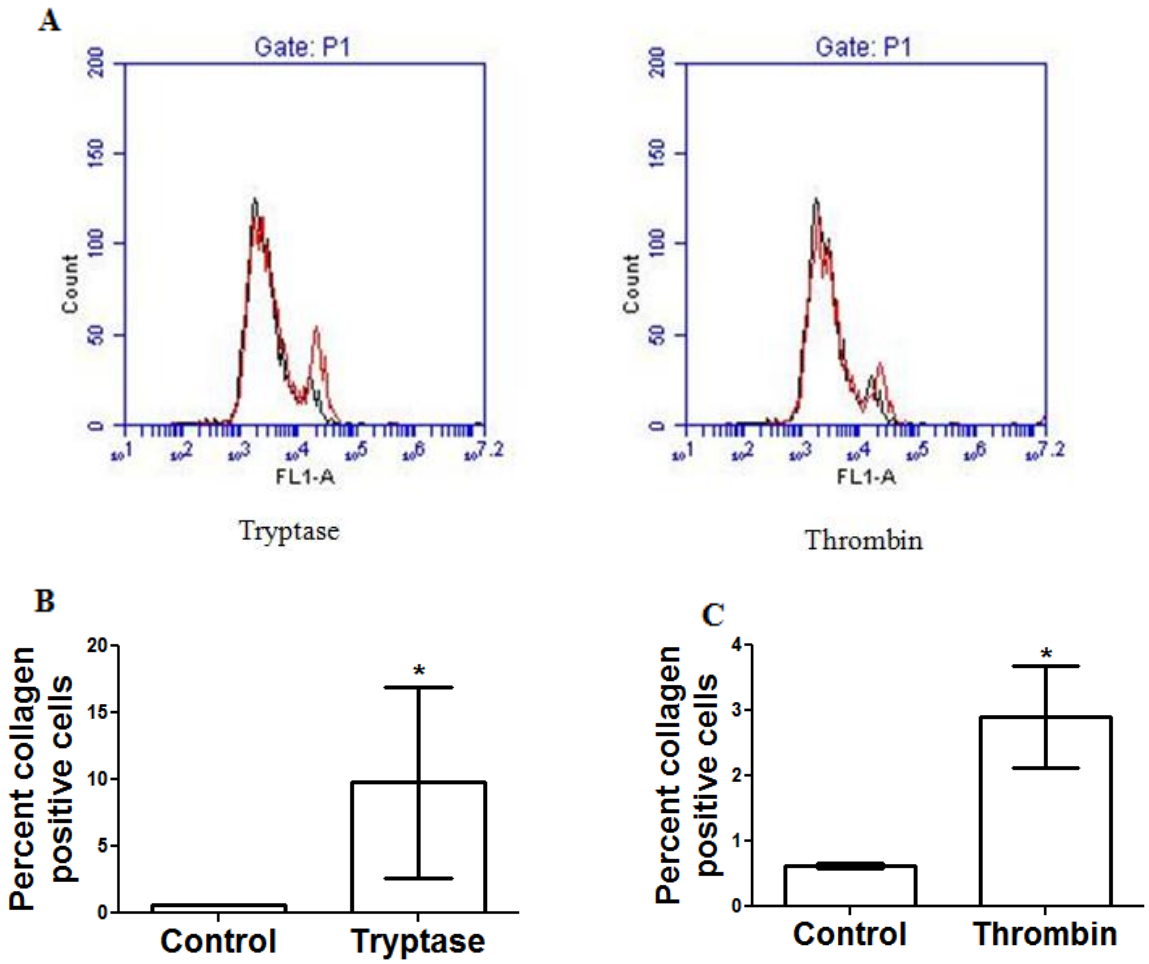

Figure 2. Tryptase and thrombin increase collagen secretion by PBMC. (A) Representative flow plots of no-protease control PBMC (black) and protease-treated PBMC (red) stained for collagen. PBMC were exposed to 12.5 ng/ml **(B)** tryptase or **(C)** thrombin for 5 days, resuspended, stained with anti-collagen and alexa-fluor secondary antibodies, and assayed for fluorescence by flow cytometry. Values are mean ± SEM, n=5, * indicates $p < .05$ by a two tailed t-test.

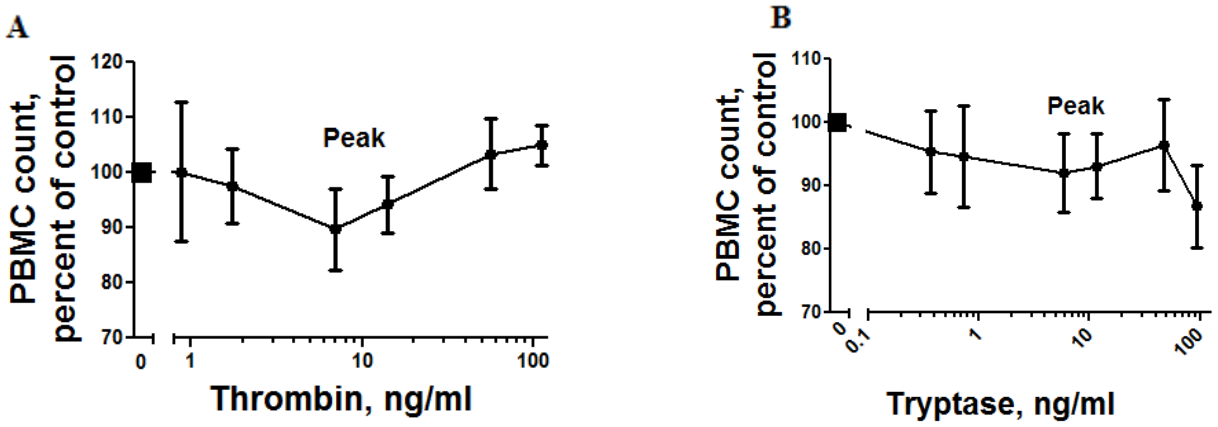

Figure 3. Thrombin and tryptase do not significantly affect the total number of adhered PBMC. The same PBMC populations in Figure 1 were then counted for the total number of PBMC adhered to the plate following fixing and staining for **(A)** thrombin and **(B)** tryptase. Counts were normalized for each donor to the no-protease control. There were no significant differences in the numbers of adhered PBMC following fixing and staining. Peak fibrocyte concentrations are labeled on the graphs.

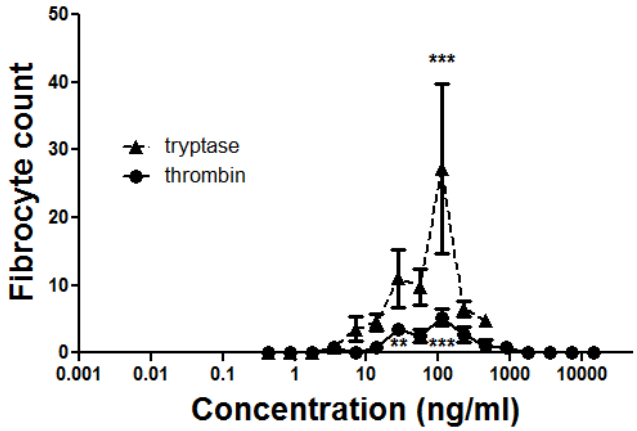

Figure 4. Thrombin and tryptase potentiate fibrocyte differentiation in the presence of human serum. PBMC were cultured in medium containing serum in the presence of the indicated concentrations of tryptase or thrombin for 5 days, and fibrocytes were counted as in Figure 1. Serum-containing media complete inhibited fibrocyte differentiation in the no-protease control. Values are mean ± SEM, n = 4. * indicates $p < .05$, ** indicates $p < .01$ and *** indicates $p < .001$ compared to the no-protease control by a two tailed t-test.

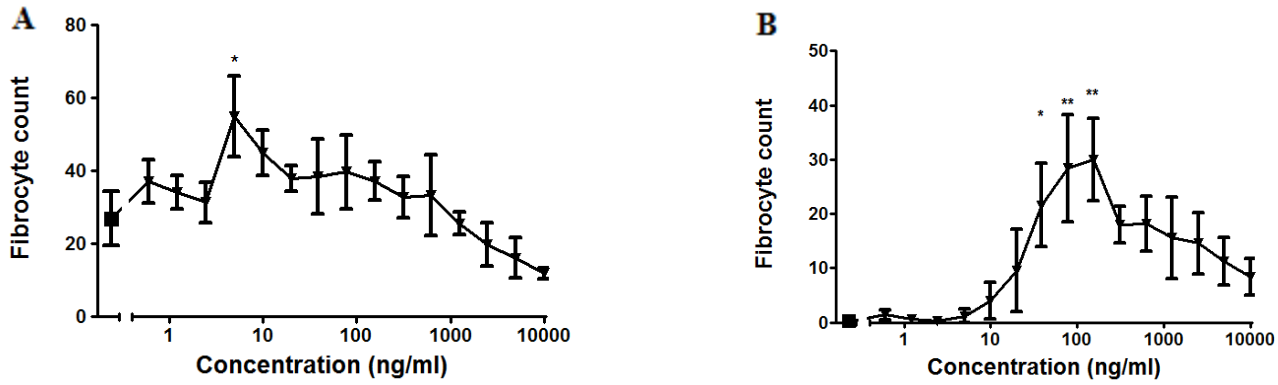

Figure 5. Trypsin potentiates fibrocyte differentiation in the presence of fish gelatin or skim milk. PBMC were cultured in the presence of 500 µg/ml **(A)** fish gelatin **(B)** or powdered skim milk in the presence of the indicated concentrations of trypsin. After 5 days, fibrocytes were counted as in Figure 1. Values are mean ± SEM, n=6. * indicates $p < .05$, ** indicates $p < .01$ compared to the no-protease control by a two tailed t-test.

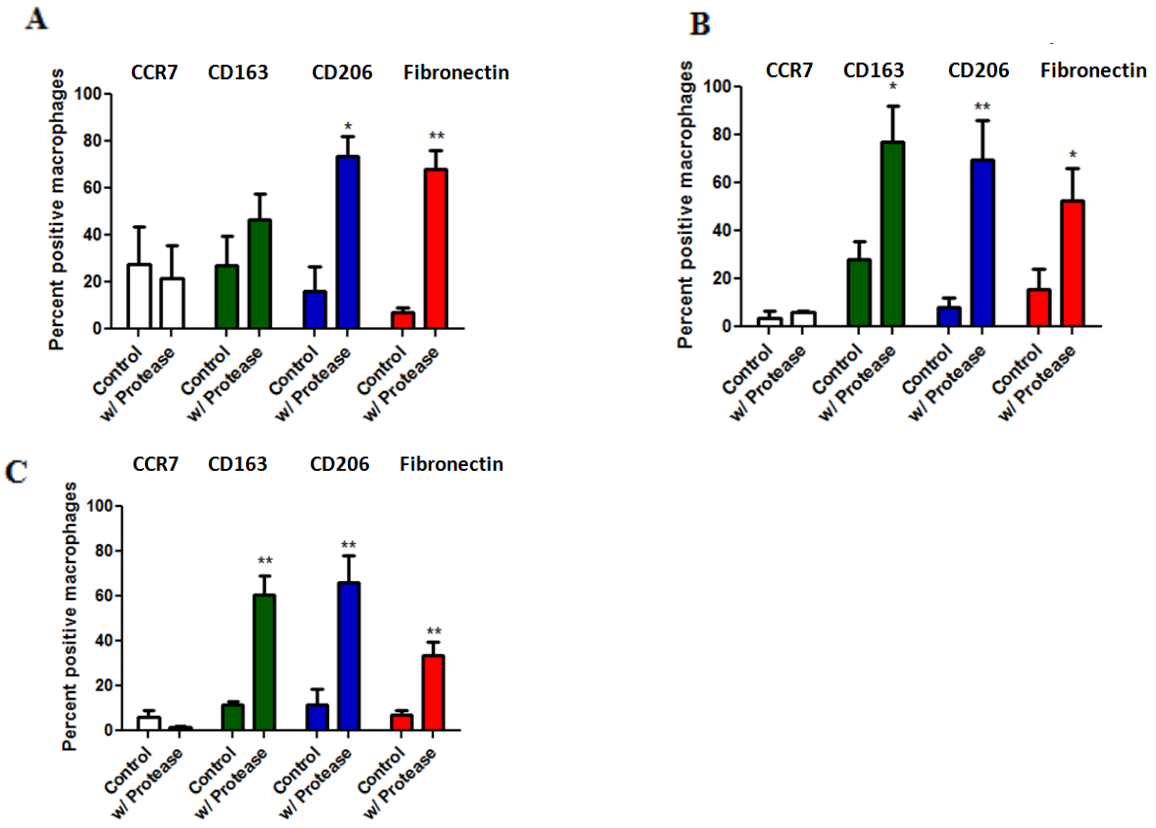

Figure 6. Trypsin, tryptase, and thrombin favor macrophage differentiation towards an M2a phenotype. PBMC were cultured in serum-free media for 5 days on 8-well chamber slides in the presence of 12.5 ng/ml **(A)** trypsin, **(B)** thrombin, and **(C)** tryptase and no-protease controls. Control and protease treated PBMC were stained by alkaline-phosphatase immunohistochemistry for CCR7 (marker of M1 macrophages), CD163 and CD206 (enigmatic markers of M2 and M2a macrophages) and fibronectin (Unambiguous marker of M2a macrophages). Stains were developed for 7 minutes. Positively stained macrophages were counted and normalized to the total number of macrophages per donor.

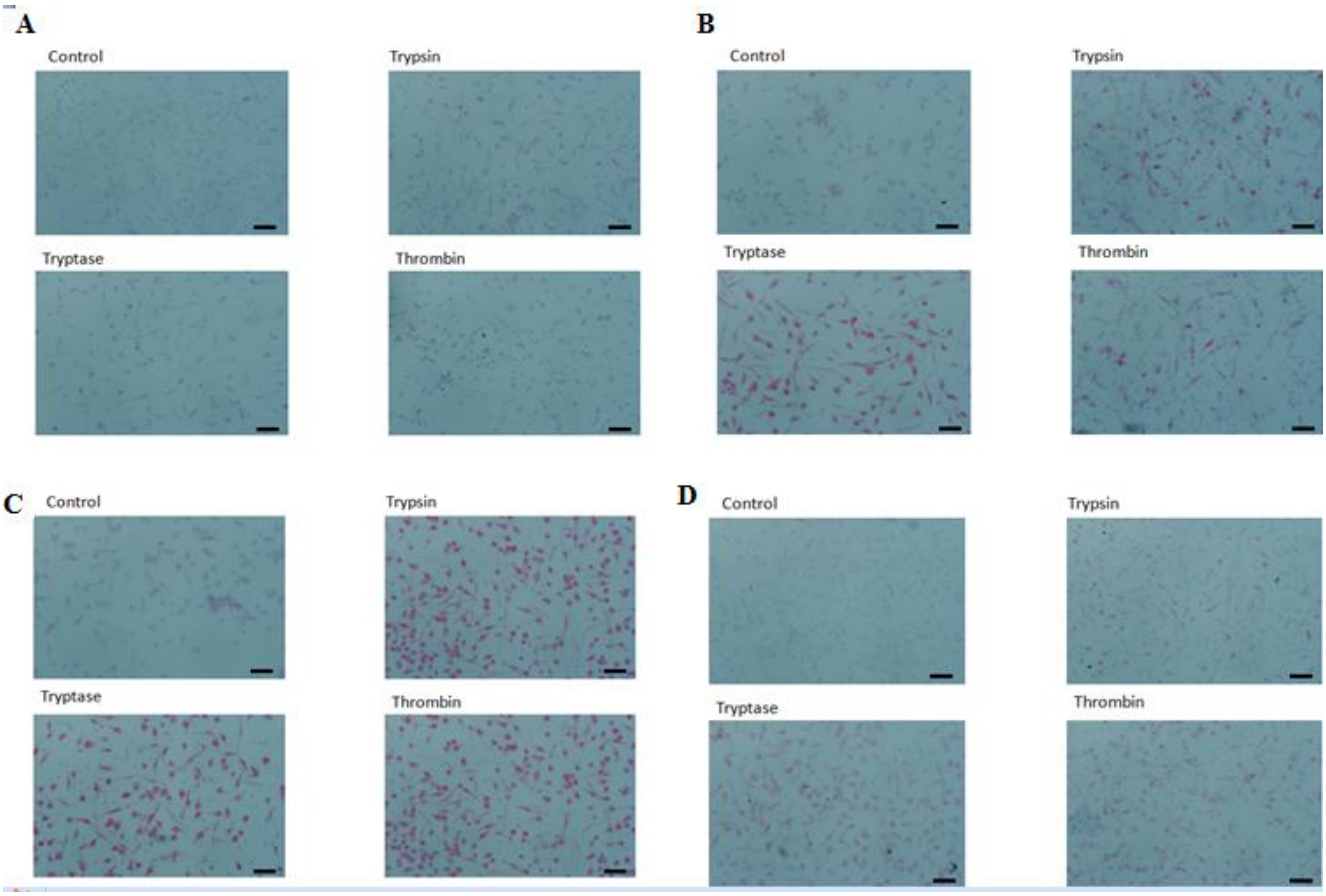

Figure 7. Trypsin, tryptase and thrombin favor macrophage differentiation towards an M2a phenotype: representative images. PBMC were cultured as in Figure 9. Representative images of immunohistochemistry on PBMC after 5 days of no-protease, trypsin, thrombin, and tryptase using antibodies for **(A)** CCR7, **(B)** CD163, **(C)** CD206, and **(D)** fibronectin. Bar is 40 µm.

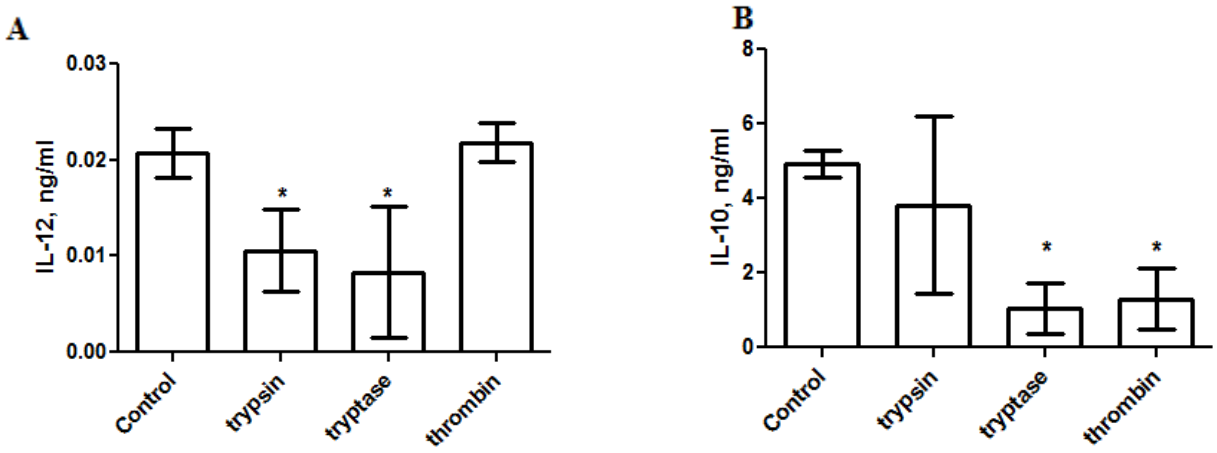

Figure 8. Trypsin, tryptase and thrombin change PBMC secretion of IL-12 and IL-10 in PBMCconditioned media. PBMC were cultured as in Figure 9. **(A)** Conditioned media from PBMC after 5 days was analyzed by ELISA for IL-12 (marker of M1 macrophages) and IL-10 (marker of M2reg macrophages). Values are mean ± SEM, n=4. * indicates $p < .05$ compared to the no-protease control by a two tailed t-test.

B

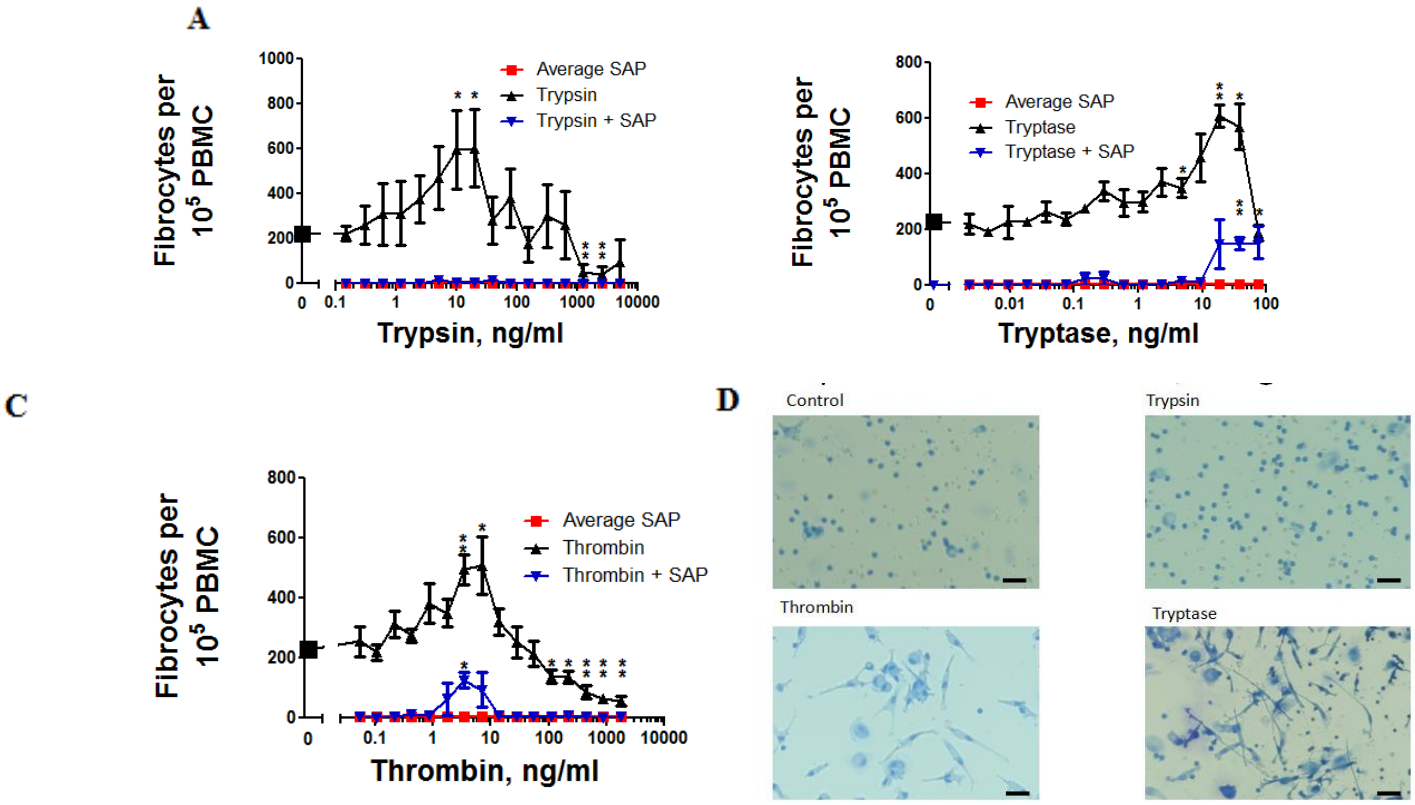

Figure 9. Tryptase and thrombin compete with SAP to potentiate fibrocyte differentiation. PBMC mixed with Serum Amyloid P (SAP) at 10 µg/ml were incubated with the indicated concentrations of **(A)** trypsin, **(B)** tryptase, or **(C)** thrombin for 5 days. Fibrocytes were then counted as in Figure 1. Values are mean ± SEM, n=4. * indicates $p < .05$ compared to proteases without SAP by t-test. **(D)** Images of PBMC after incubating for 5 days with 10 µg/ml SAP and either no protease (control), 12.5 ng/ ml trypsin, 3.5 ng/ ml thrombin, or 37.5 ng/ ml tryptase. Bar is 40 µm.

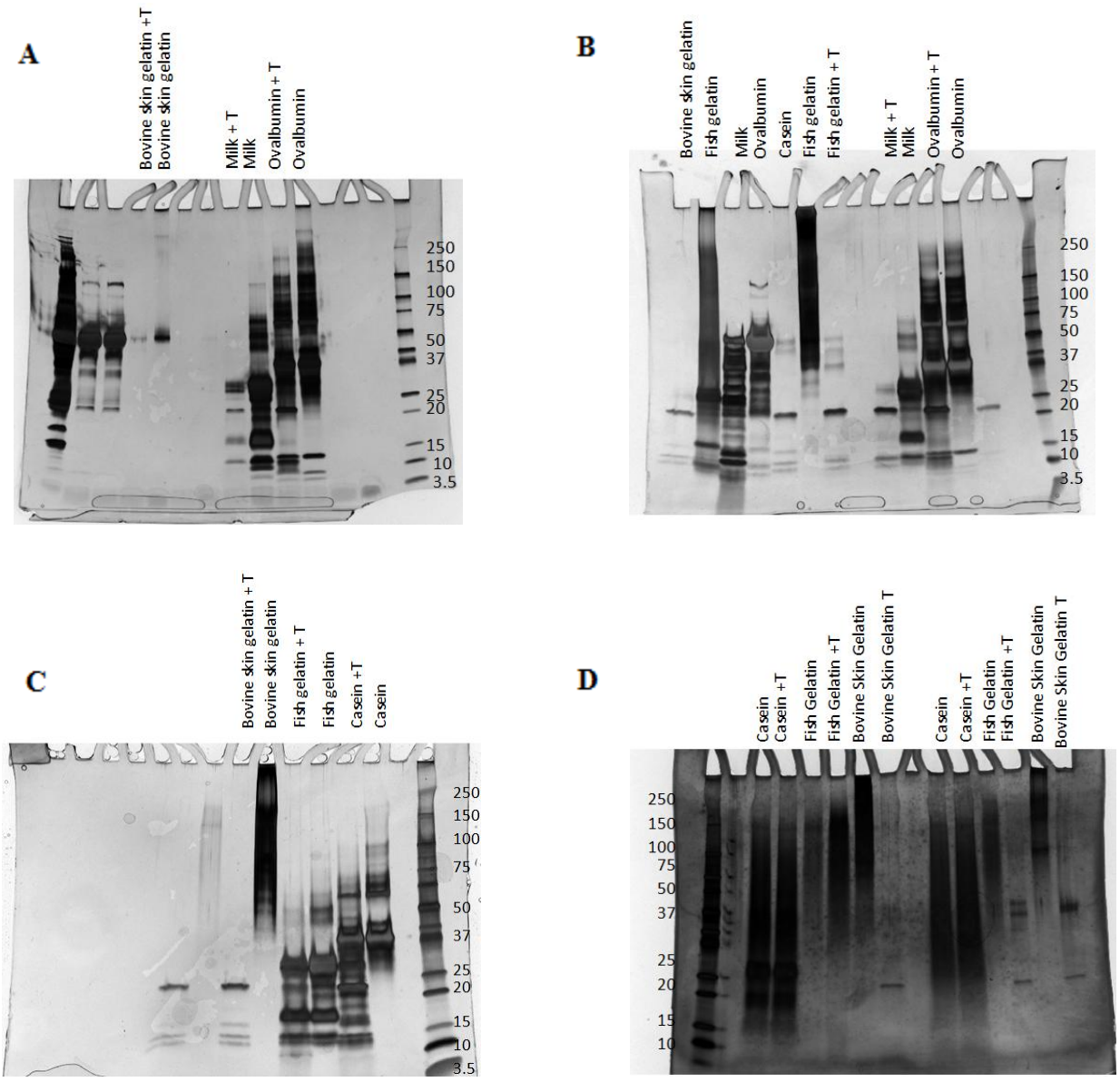

Figure 10. Trypsin cleavages media supplements

(A-C) 4-20% polyacrilamide gels were stained with silver stain, and **(D)** using a sequential stains-all stain followed by silver stain. The concentration of the supplements was 500µg/ml. Proteins were mixed with 5µg/ml trypsin, and left overnight with tumbling at 37ºC.