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PLASMINOGEN/PLASMIN AFFECTS EXPRESSION OF GLYCOLYSIS REGULATOR TIGAR AND INDUCES AUTOPHAGY IN LUNG ADENOCARCINOMA A549 CELLS

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Pericellular plasmin generation triggers apoptosis/anoikis in normal adherent cells. However, cancer cells are notoriously resistant to anoikis, enabling metastasis and new tumor growth beyond their original environment. Autophagy can be a major contributor to anoikis resistance in cancer. ***Aim:*** **To investigate if protective autophagy can be induced in lung adenocarcinoma cells in response to plasminogen treatment.** ***Materials and Methods:*** **Human lung adenocarcinoma A549 cells were incubated with Glu-plasminogen (0.1–1.0 μM) for 24 h. Pericellular plasmin activity was monitored spectrophotometrically by a cleavage of the specific chromogenic substrate S-2251. Cell survival was assessed by 3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT)-test. Degradation of fibronectin, levels of autophagy markers (beclin-1 and light chain 3 (LC3)) and glycolysis regulator (TIGAR) were evaluated by western blot. Intracellular localization of LC-3 was visualized by immunocytochemistry.** ***Results:*** **It was shown that plasminogen is converted into plasmin on the surface of adenocarcinoma cells in a dose-dependent manner. Plasmin disrupted cellular adhesive contacts resulting in cell detachment. A549 cells did not loss their viability after plasminogen treatment for 24 h, while 1.0 μM plasminogen was cytotoxic for non-transformed fibroblasts. Plasminogen 0.1, 0.5, and 1.0 μM induced 7.08-, 5.18-, and 3.78-fold elevation of TIGAR expression ($p < 0.05$), respectively. Enhanced TIGAR expression indicates switch on pentose phosphate pathway, protection against oxidative stress to prevent apoptosis, facilitation of DNA repair and the degradation of their own organelles (autophagy). Exposure of adenocarcinoma cells to plasminogen in concentrations of 0.1 and 0.5 μM caused 1.74- and 2.19-fold elevation of beclin-1 expression *vs* untreated cells ($p < 0.05$), respectively. Unlike K1–3 fragment, plasminogen treatment (0.1-0.5 μM) resulted in increased expression of LC3-I and stimulated rapid conversion of LC3-I to LC3-II. Up-regulation of beclin-1 levels and enhanced LC3-I/II conversion in plasminogen-treated A549 cells are the hallmarks of autophagy induction. According to immunocytochemistry data, increased LC3 puncta and autophagosome formation after exposure to plasminogen could reflect autophagy activation.** ***Conclusions:*** **Therefore, we showed stimulation of prosurvival signals and induction of autophagy in plasminogen-treated adenocarcinoma cells rendering them resistant to apoptosis/anoikis. Based on the obtained data, autophagy has a great potential for novel targets that affect cancer cell death, in addition to the current cytotoxic agents.**
Key Words**: plasminogen/plasmin, anoikis, autophagy, beclin-1, LC3, TIGAR.**

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Due to loss of contact with extracellular matrix (ECM) or neighbouring cells, normal adherent cells undergo an apoptotic process, termed "anoikis" (from the Greek word "homelessness"). By contrast, recent studies have shown that cancer cells acquire resistance to anoikis and can survive after detachment. Deregulation in anoikis execution contributes to the progression of malignancy and metastasis [1]. Plasmin (Pm), the active proteinase generated by plasminogen (Pg) activation on the cancer cell surface, is able to cleave adhesive glycoproteins, such as fibronectin, followed by ECM degradation and cell detachment. Pg circulates in blood plasma at the concentration of 1.5–2 μM and is also largely present in tissues in fewer amounts. Thus, Pg/Pm system represents an enormous and ubiquitous source of proteolytic activity, alterations of which contribute to tumor progression, cancer cell dissemination, invasion, and metastasis [2]. Thus, to approach the efficient prevention and treatment strategies for metastatic cancers, efforts have been made to identify key mechanisms involved in the anoikis resistance and metastatic process. Autophagy is a tightly-regulated self-degradative process that occurs at basal levels under physiological conditions in all cells and can be up-regulated in response to stressful stimuli. Autophagy produces nutrients and energy to enhance cell survival through the breakdown of cytosolic components within the autophagosomes [3]. Pro-tumorigenic functions for autophagy have been proposed [4]. Autophagy may provide a temporary survival mechanism in the condition of metabolism restriction followed by cell detachment, giving cells the chance to survive and reactivate once they reattach to the ECM [5]. Microtubule-associated protein light chain 3 (LC3) protein is the most widely used marker of autophagosome membranes [6]. Typically, autophagy induction converts LC3-I to LC3-II and enhances LC3-II level. Detection of LC3-I/II conversion by immunoblot analysis became

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Abbreviations used: Abs – antibodies; BSA – bovine serum albumin; ECM – extracellular matrix; K – kringle; LC3 – microtubule-associated protein light chain 3; MTT – 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; NADPH – nicotinamide adenine dinucleotide phosphate; PAR – protease-activated receptor; Pg – plasminogen; Pm – plasmin; RIPA – radioimmunoprecipitation assay buffer; ROS – reactive oxygen species; TIGAR – TP53-inducible glycolysis and apoptosis regulator; uPA – urokinase-type plasminogen activator; uPAR – uPA-receptor.

conventional approach because the amount of LC3-II is strongly correlated with the number of autophagosomes [7]. Beclin-1 (Atg6) is another key regulator of autophagy. Beclin 1 acts during the initiation stage of autophagy by forming the isolation membrane, a double-membrane structure that engulfs cytoplasmic material to form the autophagosome. The reduced levels of beclin 1 are thought to inhibit autophagy and prevent the turnover of damaged mitochondria, which leads to reactive oxygen species (ROS) production and genotoxic stress [8]. TP53-inducible glycolysis and apoptosis regulator (TIGAR), an important TP53-inducible glycolysis and apoptosis regulator, may play a crucial role in cancer cell survival [9]. TIGAR acts by blocking glycolysis and directing the pathway into the pentose phosphate shunt, resulting in enhanced nicotinamide adenine dinucleotide phosphate (NADPH) production needed for glutathione reduction. Therefore, increased expression of TIGAR protects cells from oxidative-stress induced apoptosis by decreasing the ROS levels [10].

It is known that lung cancer is the leading cause of cancer-related deaths in the world with non-small cell lung cancer making up about 85% of all lung cancer cases [11]. Adenocarcinoma A549 cell line is one of the most commonly used human non-small cell lung cancer cell lines for basic research and drug discovery [12]. These cells have relatively high proteolytically active phenotype by overexpressing proteins of Pg activation system, including surface-bound urokinase-type plasminogen activator (uPA)/uPA-receptor (uPAR) cascade [13]. Thus, the aim of the present study was to investigate if plasminogen treatment is able to trigger autophagy, representing a plausible mechanism of overcoming cell death in lung adenocarcinoma cell line in response to plasmin-induced detachment.

MATERIALS AND METHODS

Purified proteins. Human Glu-Pg was purified from the fresh blood plasma by affinity chromatography on lysine-sepharose column as described earlier [14] and was considered to be 99% pure as assessed by sodium dodecyl sulphate polyacrilamide gel electrophoresis and did not display spontaneous proteolytic activity. Elastase-derived Pg fragment, known as angiostatin, which contains the first three kringle (K) domains (K1–3) and lacks serine-proteinase domain, was prepared according to Sottrup-Jensen *et al*. [15].

Cells and experimental design. The lung adenocarcinoma A549 cells (ATCC, Rockville, MD, USA) were cultivated in DMEM with 10% fetal bovine serum (Sigma-Aldrich, USA) at 37 °C in humidified atmosphere with 5% CO_2. Then, A549 cells were incubated with Glu-Pg (0.1–1.0 μM) or, in some experiments, with fragment K1–3, at 37 °C for 24 h in serum-free DMEM.

Plasmin activity assay. Glu-Pg (0.1–1.0 μM) or K1–3 (1.0 μM) were added to A549 cells grown in 96-well plate (2.5×10^4 cells/well) and mixed. Then, chromogenic substrate S-2251 was added and mixed. The absorbance changes were monitored at 405–492 nm for 90 min by microreader Titertek Multiskan MC (Finland). All measurements were performed in triplicate.

3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT)-test. MTT reagent was added to A549 cells grown in 96-well plate (2.5×10^4 cells/well) treated with Glu-Pg (0.005–1.0 μM). Non-transformed embryonic fibroblasts 3T3 (ATCC, Rockville, MD, USA) were used as a control. After 24 h of incubation, plates were centrifuged in a cytospin centrifuge to sediment detached cells, as described before [16]. Formazan crystals were dissolved in dimethyl sulfoxide. Absorbance was determined by micro-reader at 550–620 nm. Each measurement was performed in six replicates.

Western blot. Whole-cell extracts were prepared in radioimmunoprecipitation assay buffer according to the standard instructions. Extracts (20 μg protein per track) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes by electroblot. Following blocking in 5% non-fat dry milk solution, membranes were probed with individual antibodies (Abs) that were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA): mouse anti-fibronectin EP5 (sc-8422), mouse anti-TIGAR (sc-377065), mouse anti-beclin-1 (sc-48341), and rabbit anti-LC-3 (sc-134226). Membranes were washed and further probed with an appropriate horseradish peroxidase-conjugated secondary Abs. After incubation with the HRP substrate, membranes were exposed on X-ray film (Konica Minolta, Japan), immunostaining was developed by enhanced chemiluminescence with the use of automatic device (Carestream Medical X-Ray Processor 2000, USA). Signals were visualized, digitized, and analyzed using TL120 software (TotalLab Ltd., USA). Molecular weights were determined using standard prestained transblot molecular weight markers (PageRuler, cat. no. 26616, Fermentas, Germany). Band density was normalized to tubulin used as a loading control. The results were expressed as units of density × band area.

Immunocytochemistry assay and confocal scanning laser microscopy. For detection of autophagy flux, we used immunocytochemistry for LC3 in A549 cells. Briefly, cells were seeded on coverslips in 6-well plates in DMEM medium. The cells were then treated with Glu-Pg (0.1–1.0 μM), K1–3 (1.0 μM) or vehicle control for 24 h. After medium removing and washing, the cells were fixed with 4% buffered paraformaldehyde solution for 20 min at room temperature. After washing, coverslips were dried and cells were permeabilized with ice-cold acetone. Samples were then blocked using 5% bovine serum albumin (BSA) solution for 90 min at 37 °C. Primary anti-LC3 Abs were diluted 1/100 in 1% BSA and 0.3% Triton X-100. The blocking solution was removed, and the primary Abs solution was added to the samples and incubated overnight at 4 °C. After incubation, primary Abs solu-

tion was removed, cells were washed, and secondary anti-rabbit FITC-conjugated Abs (Sigma-Aldrich, USA) 1/250 diluted in 3% BSA were added to the samples and incubated for 3 h at 37 °C in the dark. To counterstain nuclei, the solution of Hoechst 33342 (Sigma-Aldrich, USA) (1 μg/mL) was added to the samples and incubated for 5 min at room temperature in the dark. Confocal microscopy with the use of LSM510 (Zeiss, Jena, Germany) was performed right after the samples were stained.

Statistical analysis. All variables were expressed as mean ± standard error of mean or as medians in the case of non-normal distribution mean. The differences between the groups were tested by one-way ANOVA followed by post hoc Tukey test, using OriginPro 8.6 (OriginLab Corp., USA). The confidence interval (p) less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Adenocarcinoma A549 cells convert Pg into Pm. Cultured A549 cells can convert Pg into Pm in a time- and dose-dependent manner even in the absence of exogenous activators (Fig. 1). Neither control cells nor K1–3-treated cells displayed Pm activity unless exogenous Pg was added. These data indicate that, in the cell system, used in our experiments, constitutively expressed Pg activators appeared to be responsible for pericellular Pg activation and the subsequent Pm generation. Literature data indicate that cancerous lung epithelial cells abundantly express uPA/uPAR, which is critical for cellular migration and tissue remodelling following either lung injury or the propagation and metastasis of lung neoplasms [17].

Cell viability. We treated both the A549 cell line and 3T3 fibroblasts with Pg (0.005–1.0 μM) for 24 h, and cell viability was examined by MTT assay. Keeping in mind that Glu-Pg occurs in plasma in concentration up to 2 μM, the chosen dosage is more related to the amounts of this protein in ECM.

As shown in Fig. 2, absence of reduction in cell viability was observed for cancerous epithelial A549 cells after 24 h of exposure to Pg in the studied concentration range. However, a significant capacity to kill normal 3T3 cells was detected at a Pg concentration as high as 0.75 μM. It is worth-noting that cells appeared to be detached in a dose-dependent manner as a result of such treatment, and changes in their morphology (from elongated spindle- to round-shaped) were seen by light microscopy (data not shown).

Pm-induced fibronectin degradation. To investigate the relation between Pm formation and ECM protein degradation, we evaluated the extent of fibronectin fragmentation by western blot (Fig. 3). We found

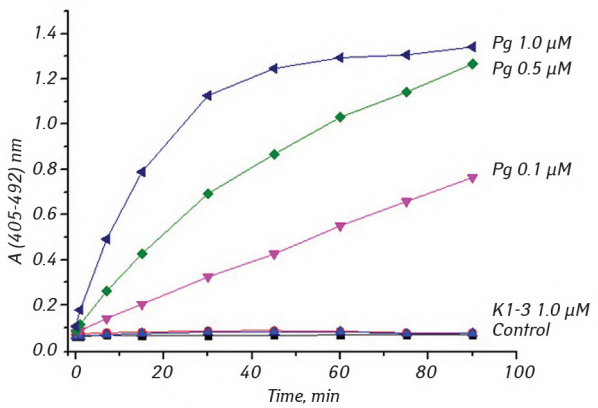

Fig. 1. Kinetics of plasminogen activation by adenocarcinoma A549 cells

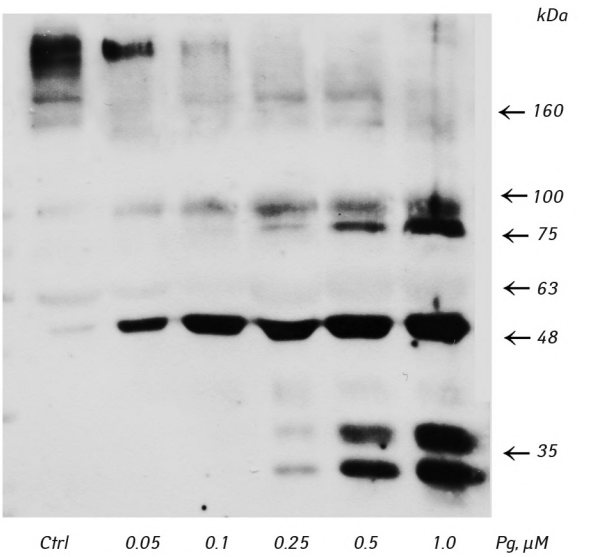

Fig. 3. Western blot of fibronectin and its proteolytical-derived fragments in lysates of A549 cells treated with plasminogen

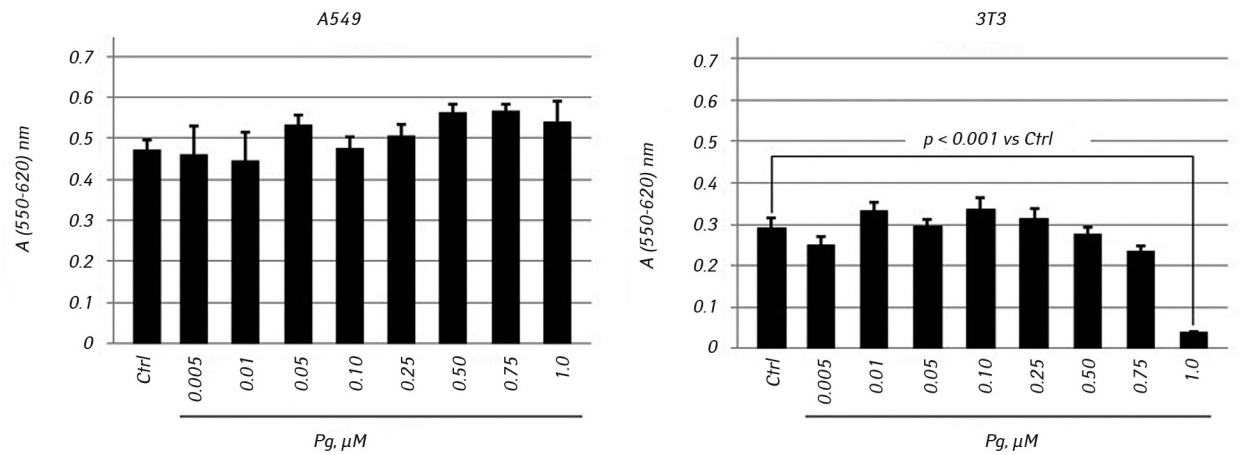

Fig. 2. Effects of plasminogen/plasmin on viability of A549 and 3T3 cells during 24 h exposure

that fibronectin underwent degradation as a result of Pg exposure.

Pm cleaved fibronectin to multiple degradation products, each less than 200 kDa (180–25 kDa). Complete disappearance of the fibronectin monomer subunit (~ 220 kDa) together with the generation of the 50–25 kDa fragments at Pg concentration as high as 0.25 μM were observed. Thus, fragmentation of fibronectin can occur in a result of Pg activation and Pm-mediated proteolysis of this ECM protein.

Pg treatment up-regulates TIGAR expression. Results of western blot, presented in Fig. 4, indicate that 0.1, 0.5, and 1.0 μM Pg induced 7.08-, 5.18-, and 3.78-fold elevation of TIGAR expression in A549 cells ($p < 0.05$ *vs* control), respectively.

Pg treatment induces autophagy in A549 cells. Western blotting analysis was performed with some of the proteins associated with the autophagic pathway, such as beclin-1 and LC3. As shown in Fig. 5, Pg 0.1 and 0.5 μM caused significant ($p < 0.05$ *vs* control) increase of an autophagy marker, beclin-1 in lung adenocarcinoma A549 cell line (1.74- and 2.19-folds, respectively). It is of interest, that like in the case of TIGAR expression, the maximal studied Pg concentration resulted in weaker changes in protein levels.

The same tendency was observed for the expression of another autophagy indicator, LC3, suggesting that lower concentrations of Pg exert activated effects toward A549 cells (Fig. 6). Reversal dose-response effect reflecting in apparent gradual decrease in LC3 levels caused by increasing Pg concentrations could be due to its intense lysosome-dependent degradation after formation of autolysosomes [18].

In contrast to K1–3 fragment, plasminogen treatment (0.1–0.5 μM) resulted in the enhanced expression of LC3-I and stimulated rapid conversion of LC3-I to LC3-II. Up-regulation of beclin-1 levels and enhanced LC3-I/II conversion in plasminogen-treated A549 cells are both the hallmarks of autophagy initiation [19]. LC3 is a marker protein that is involved in the formation of autophagosomes and autolysosomes, which are usually characterized and monitored by fluorescence microscopy [7]. Immunocytochemistry data, shown in Fig. 7, confirm western blot results and demonstrate increased LC3 puncta and autophagosome formation after Pg exposure, which could reflect autophagy activation.

LC3 is processed at its C-terminus by Atg4 and becomes LC3-I just after synthesis. LC3-I is subsequently conjugated with phosphatidylethanolamine to become LC3-II. LC3-II associates with both the inner and the outer membranes of the autophagosome and therefore it has become a good marker for autophagy flux [6]. LC3 puncta was visualised by fluorescence microscopy as a diffuse cytoplasmatic pool in untreated cells and cells treated with 0.1 μM Pg, and as punctate structures representing autophagosomes in cells treated with 1.0 μM.

Lung cancer is the most common cancer and the leading cause of cancer-related deaths world-

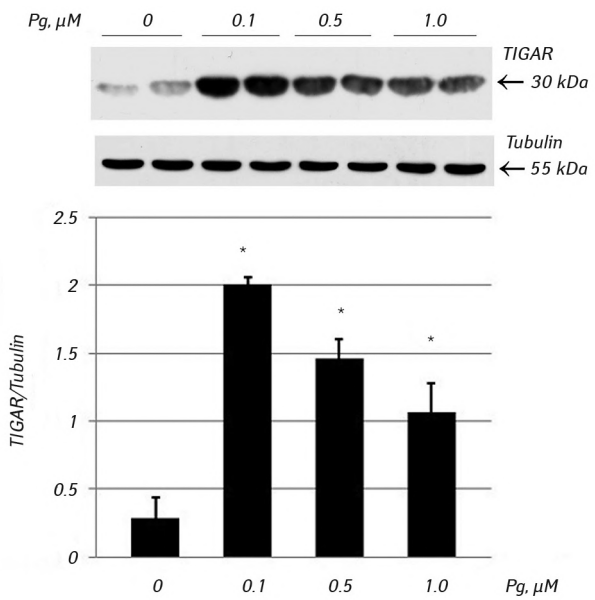

Fig. 4. Expression of TIGAR in A549 cells treated with plasminogen (*$p < 0.05$ compared to control)

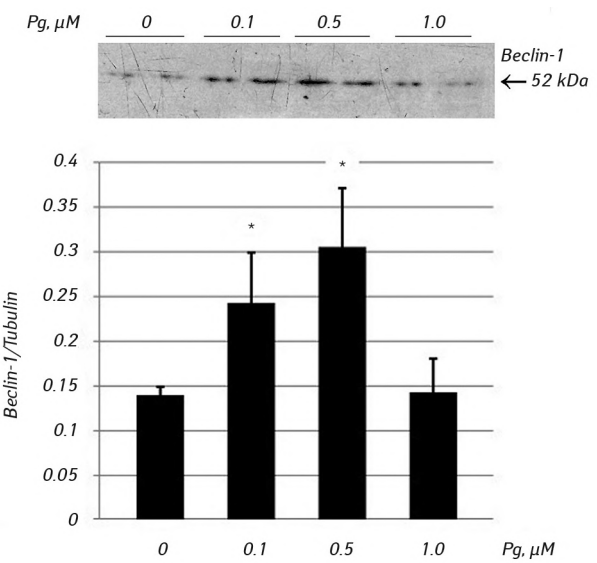

Fig. 5. Expression of beclin-1 in A549 cells treated with plasminogen (*$p < 0.05$ compared to control)

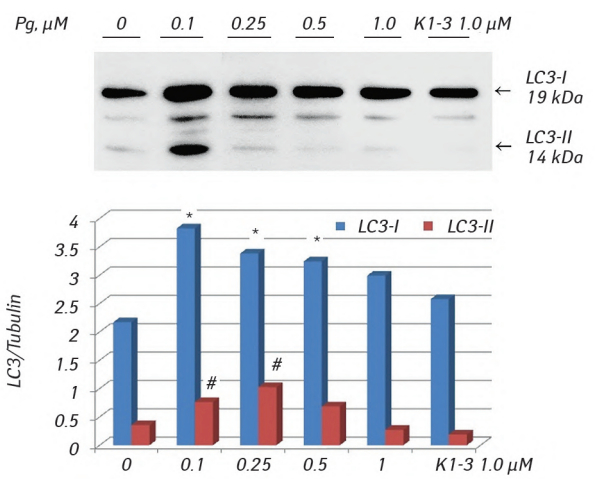

Fig. 6. Expression of LC3 in A549 cells treated with plasminogen (*$p < 0.05$ compared to control for LC3-I; #$p < 0.05$ compared to control for LC3-II)

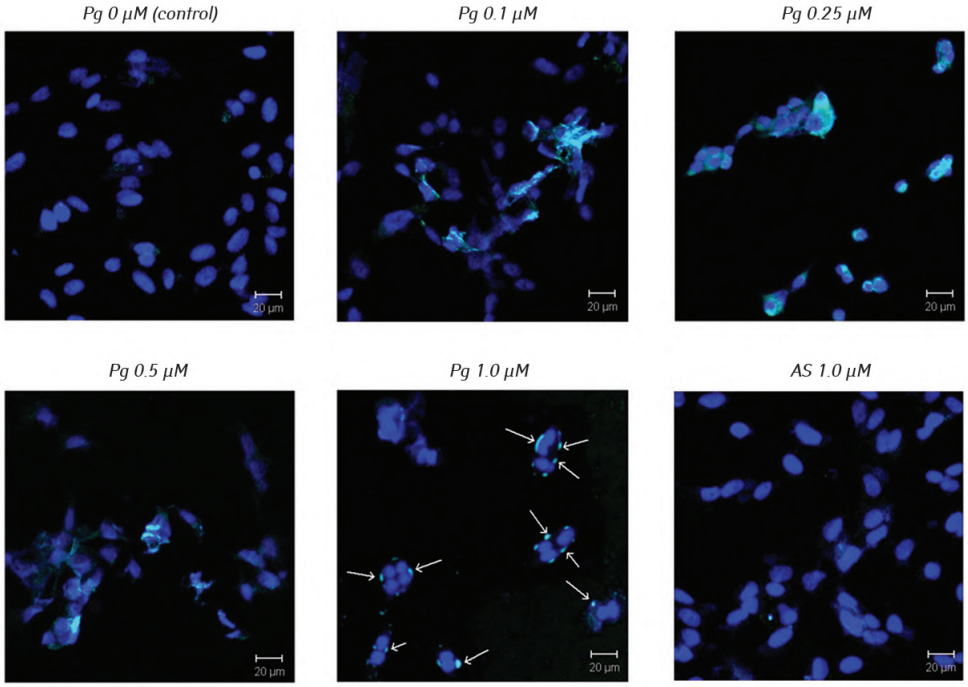

Fig. 7. Immunofluorescence staining of LC3 in A549 cells treated with Pg or angiostatin K1-3 (green — specific LC3 staining, blue — nuclei, arrows mark autophagic puncta)

wide [11]. Metastasis, a spread of cancer cells to secondary sites, is a major cause of cancer-related death which frequently occurs in lung cancer patients [20]. Detailed molecular and functional analyses of anoikis resistant cells may provide insight into the biology of cancer metastasis and identify novel therapeutic targets for the prevention of cancer dissemination. A rupture of the balance between the Pg activators and inhibitors can be a starting point for the local generation of Pm. Since Pg can become membrane bound, the occurrence of receptors for Pg and uPA on the same cell results in the formation of surface-associated Pm, which degrades fibrin, adhesive proteins, and ECM components, such as fibronectin, vitronectin, laminin, and fibrinogen. It can also activate matrix metalloproteinases and controls bioavailability of some growth factors [2]. These events are associated with degradation and remodelling of the surrounding tissues and are also fundamental to tumor progression, expansion of the tumor mass, induction of tumor cell proliferation, invasion, migration, and release of tumor growth factors and cytokines [21]. We showed that Pm indeed appeared to be the enzyme responsible for fibronectin cleavage. In turn, Pm-dependent cleavage of pericellular proteins results in cell retraction and detachment, leading to apoptosis of adherent cells, a process called anoikis. It has been shown earlier that surface-bound Pm triggers apoptosis/anoikis of aortic valvular myofibroblasts [22] and smooth muscle cells [23]. To metastasize, tumor cells must have an ability to overcome anoikis or apoptosis induced by loss of cell adhesion [1]. However, mechanisms of apoptosis/anoikis avoidance by cancer cells, which promote both survival and migration, are poorly explored. It is supposed that mechanisms of stimulation of both prosurvival signals and suppression of death signals are involved in anoikis resistance. From this point of view, autophagy can function as either a pro-death or pro-survival depending on the context and the stimuli. However, these roles are hard to identify since autophagic activity in dying cells can be an attempt to survive [4]. In our work, we confirmed the hypothesis that autophagy activation may represent a response to proteinase-mediated stress and may provide temporal survival of the detached cancer cells. Pm generation on the surface of A549 cell and loss of cell adhesion to ECM lead to up-regulation of definitive autophagy-associated proteins, beclin-1 and LC3, indicating autophagy activation and autophagosome formation. There are several lines of evidence that beclin-1-mediated autophagy inhibits apoptosis and promotes survival during stress [8, 24]. For example, expression of beclin-1 in human MCF-7 breast carcinoma cells promotes autophagy [25]. On the other hand, autophagy may also play a role in programmed cell death [5]. However, we suggest that Pm, acting in the chosen concentrations (up to 1.0 μM), induces autophagic survival response in A549 cells, which allows them to overcome stringent conditions thereby extending the life span, in contrast to non-transformed embryonic fibroblasts. Intriguingly, some isolated K domains of Pg/Pm, which are lack of proteolytic activity, appear to be able to induce autophagy. Nguyen *et al*. [26] have earlier reported that isolated K5 evokes an autophagic response in endothelial cells that is specific and initiated even in the absence of nutritional stress. Endothelial cells exposed to K5 up-regulated beclin-1 levels within a few hours. Therefore, the plausible role of K domains as inductors of autophagy in tumor cells needs to be further explored.

The role of cancer cell's protease-activated receptors (PARs), a group of G-protein-coupled receptors, in transmitting signals in response to extracellular protease action is extensively highlighted [27]. A549 cells express all four types of PARs (PAR-1, -2, -3, and -4) [28]. PAR-1 and PAR-2 were identified to be widely involved in tumor progression [29]. Previous studies showed that the up-regulation of PAR-1 is strongly associated with low survival rates in patients with gliomas, breast cancer, and primary gallbladder carcinoma, while inhibition of PAR-1 is beneficial to patients with tumors [30]. Huang *et al*. [31] have demonstrated that activation of PAR-2 decreases the ratio of Bax/Bcl-xL and reduces apoptosis in A549 cells. Pericellular Pm has been demonstrated to cleave and activate PARs with various rates and efficacies, preferentially PAR-1 and -4, and may in this way provide powerful protective mechanisms for cancer cells [32]. Moreover, proteolytical activation of PAR-1 by Pm is critical for Pm-induced cell migration [33].

Overactivation of PAR-dependent pathways, for example PAR-1/NADPH oxidase, may result in enhanced intracellular ROS production [34]. Although the role of ROS in cancer cell metastasis and its regulation are largely unknown, excessive amounts of ROS can be damaging to the cell, inducing DNA damage, and lipid oxidation. Ultimately, left unchecked, ROS can trigger apoptosis and cell death [35]. ROS-activated signalling can be attenuated by antioxidant systems. In response to enhanced ROS generation, antioxidant enzymes, including those of the glutathione system, consume NADPH to reduce ROS-induced damage [36]. Recently, TIGAR has been shown to function to hydrolyse fructose-2,6-bisphosphate and fructose-1,6-bisphosphate, two activities that lead to the same effects on glycolysis [37]. Expression of TIGAR results in a decreased glycolytic rate. This leads to the redirection of glycolytic metabolic intermediates to the oxidative branch of the pentose phosphate pathway. One consequence of this function of TIGAR is an increased NADPH production, which contributes to the scavenging of ROS by reduced glutathione. Induction of this pathway by TIGAR results in decreasing intracellular ROS levels and lowering sensitivity of cells to oxidative stress-associated apoptosis. From these circumstances, TIGAR is able to protect cancer cells against ROS-induced apoptosis and to induce DNA damage repair [9, 10]. Accumulating evidence shows that TIGAR is highly expressed in many types of cancers, including leukemia, breast, lung, liver, and colon cancers [38]. So, TIGAR is a potent mediator of tumor progression and may serve as a molecular target of cancer pharmacotherapy. Up-regulation of TIGAR expression in Pg-treated A549 lung cancer cells may be accounted for as a novel protective mechanism against Pm-induced cytotoxicity.

CONCLUSION

We found a plausible link between proteinase-mediated stress, autophagy, and regulation of central metabolic pathways. Induction of autophagy and deregulation of apoptosis machinery in lung adenocarcinoma cells render them resistant to apoptosis/anoikis. Based on the obtained data, plasmin-induced autophagy might have a great potential as a supplementary target to affect cancer cell death, in addition to the current cytotoxic agents. Studies by authors of this report to evaluate potential etiologic relationships between these processes are ongoing.

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