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Regulation of Zn-α2-Glycoprotein-Mediated Cell Adhesion by Kininogens and Their Derivatives

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MC3T3-E1 (mouse osteoblast-like) cells adhered to a tissue culture plate coated with human Zn-α2-glycoprotein (Znα2gp). The adhesion of MC3T3-E1 cells to Znα2gp was inhibited by synthetic peptides such as RGDS and ELRGDV, and by antibody against vitronectin receptor. These findings suggested that the RGD region of Znα2gp interacts with the vitronectin receptor (αvβ3) on the MC3T3-E1 cell surface. Furthermore, we found that the common heavy chain of both HMW- and LMW-kininogens accelerated the Znα2gp-mediated MC3T3-E1 cell adhesion. Among the three domains of the common heavy chain of both kininogens, domain 3 promoted the cell adhesion by up to 200%. Among the nine synthetic peptides covering domain 3, the peptide, N^{334}AEVYVVPWEKKIYPTVN^{351} accelerated in a dose-dependent manner the Znα2gp- and vitronectin (VN)-mediated MC3T3-E1 cell adhesion. These findings suggested that a defined region of domain 3 is responsible for the acceleration of cell adhesion.

Key Words: **Zn-α2-glycoprotein; cell adhesion; high- and low-molecular weight kininogens; domain 3.**

Zn-α2-glycoprotein (Znα2gp), with a molecular weight of 38,000-41,000, was first isolated from human blood plasma (1). We reported structural differences between the blood plasma and seminal plasma forms: the seminal plasma protein lacked an N-terminal amino acid (pyroglutamic acid), and contained no carbohydrate (2,3). The distribution of Znα2gp indicated that the protein is immunohistochemically localized in the grandular exocrine epithelium of various human tissues including mammary gland, prostate, and saliva, besides the hepatocytes and the epithelial cells of the proximal and distal tubules in the kidney (4). As to its physiological function, Shibata *et al.* (5) suggested that Znα2gp is a carrier protein of nephritogenic renal glycoprotein(s). Araki *et al.* (6) showed that the amino acid sequence of Znα2gp is similar to those of major histocompatibility antigens (HLA class I-α chain and HLA class II), and speculated that the protein plays a role in the immune response. We predicted the whole amino acid sequence from human prostate cDNA coding Znα2gp (7). In the amino acid sequence of the protein, we found a consensus cell adhesion sequence (Arg-Gly-Asp) which plays an important role in cell adhesion, and reported a novel physiological function of Znα2gp as an adhesive protein using SMKT-R3 (8).

Asakura *et al.* (9) reported that kinin-free high molecular weight kininogen (kf-HK) strongly inhibits adhesion of MG-63 (human osteosarcoma) cells to vitronectin-coated plate, and suggested that the f1 · 2 region is resposible for the inhibition.

Herein, we report that the MC3T3-E1 (mouse osteoblastic-like) cells adhered to the Znα2gp-coated surface and show that the vitronectin receptor takes part in Znα2gp-mediated cell adhesion. We provide evidence that low molecular weight-kininogen (LK) and its derivatives promote Znα2gp-mediated MC3T3-E1 cell adhesion, and that domain 3 of the common heavy chain of both kininogens is responsible for the accelaration. Furthermore, we identified the amino acid sequence accelerating Znα2gp-mediated MC3T3-E1 cell adhesion.

MATERIALS AND METHODS

Materials. Human seminal plasma and plasma Znα2gp were purified as previously reported (2,3). Human plasma fibronectin (FN) and vitronectin (VN) were also purified by the methods of Hayashi *et al.* (10), and Yatogoh *et al.* (11), respectively. Human laminin (LN) was purchased from Becton Dickinson Labware (Bedford, MA, USA). Peptides were synthesized with an automated peptide synthesizer (Model 431A, Applied Biosystems, Foster City, CA, USA) using Fmoc chemistry, and purified using a reverse phase column in an HPLC

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system. Low molecular weight kininogens (LK), kinin-free high molecular weight kininogen (kf-HK), kinin- and f1 · 2-free HK(α1-TPI), kinin-free LK (α2-TPI), domains 1, 2 and 3, and light chains of both kininogens were purified by the methods of Ohkubo *et al.* (12,13) and Higashiyama *et al.* (14). MC3T3-E1 cells (15,16) were obtained from Riken Cell Bank (Tsukuba, Japan). α-Minimum essential medium (α-MEM), heat inactivated fetal bovine serum and trypsin-EDTA (0.05% Trypsin, 0.53 mM EDTA-4Na) were obtained from GIBCO BRL (Gaithesburg, MD, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazorium bromide (MTT) and fluorescein isothiocyanate (FITC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 96- and 24-well tissue culture plates were obtained from IWAKI GLASS (Chiba, Japan). Rabbit anti-human vitronectin receptor (VNR) and fibronectin receptor (FNR) polyclonal antibodies were obtained from CHEMICON INTERNATIONAL INC (Temecula, CA, USA).

Cell adhesion assay. Wells of a 96-well tissue culture plate were coated overnight at 4°C with 1 μg/100 μl/well of adhesive protein. Nonspecific absorption sites were blocked with 150 μl (1.5 mg/well) of heat-denatured bovine serum albumin. The plate was washed with phosphate-buffered saline (PBS) several times. Cells used for assays were obtained from subconfluent cultures in log-phase growth, and were released from stock monolayers by incubation for 3 min at 37°C with trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA-4Na). The cells then were resuspended in α-MEM. 3.5×10^4 Cells/well in the α-MEM (100 μl) were added to each well of a 96-well tissue culture plate. The cells were incubated for 60 min at 37°C in a 5% CO_2 incubator. Non-adherent cells were removed by washing with α-MEM. The number of spreading cells was determined visually with a microscope. The adherent cells were determined with an MTT assay system as described by Takagaki *et al.* (8).

FACS analysis. To analyze the expression of the integrins $\alpha5\beta1$ and $\alpha v\beta3$, MC3T3-E1 cells were incubated with anti-human integrin $\alpha5\beta1$ or anti-human $\alpha v\beta3$ rabbit polyclonal antibody (10 μl) for 20 min on ice. After washing twice with α-MEM, the cells were stained with goat anti rabbit IgG conjugated with FITC for 20 min on ice. After washing twice again with α-MEM, the stained cells were resuspended in α-MEM, and applied onto FACScan (Becton Dickinson, Mountain View, CA, USA).

To analyze the binding strength of kininogens and their derivatives, and peptide 8 (P-8) and peptide 8s (P-8s) which scrambled the amino acid sequence of peptide 8, to MC3T3 E-1 cells, these proteins and peptides were labeled with FITC essentially as described (17) Briefly, each protein (0.5 mg) or peptide (1mg), and 0.1 mg of FITC in 0.2 ml of 0.5 M $NaHCO_3$, pH 9, was incubated overnight at 4°C in the dark. Free FITC was removed using a Sephadex-G25 or Bio-Gel P-2 column. The concentration of FITC-labeled proteins were determined using a Bio-Rad Protein assay kit (Bio-Rad Lab., Hercules, CA, USA). FITC-labeled proteins and peptides were added to MC3T3 E-1 cells at a final concentration of 0.6 μM and 25 μM, respectively. The mixture was incubated for 20 min at room temperature, and then the protein or peptide bound to the cells was quantified by FACScan.

Protein concentration. Protein concentrations were determined with the following extinction coefficients ($E^{1\%}_{280nm}$):18.0 for Znα2gp (1); 12.8 for FN (18); 13.8 for VN (19), 7.3 and 7.8 for HK and LK (20) and 7.8 for α2-TPI (13), 6.5 for α1-TPI (13) and 10.0 for domains 1, 2 and 3.

RESULTS

Assay Conditions, Cells and Receptors for Znα2gp-Mediated Cell Adhesion

Suitable conditions for the cell adhesion including concentration of Znα2gp, cell numbers and incubation time were decided on: 1 μg/well, 3.5×10^4 cells/well and 90 min, respectively. Among various established cell lines which associated with bone formation and osteosarcoma, such as MC3T3-E1, MG-63, Ros-17/2.8-5 (rat ostesarcoma) and OST (human osteosarcoma), only MC3T3-E1 significantly adhered to the seminal plasma or plasma Znα2gp-coated plate. Synthetic peptides RGDS and ELRGDV inhibited the MC3T3-E1 cell attachment and spreading in a dose dependent manner (IC_{50} = 3 μM), whereas ELRGEV and GRGESP did not. Furthermore, the strength of MC3T3-E1 cell adhesion to the protein coated plate was in the order of FN > LN > Znα2gp $\geq$ VN $\geq$ osteopontin (OPN).

To clarify the effect of VN and FN receptors (VNR and FNR) on Znα2gp-mediated cell adhesion, anti-human VNR or FNR rabbit polyclonal antibody was used. The MC3T3-E1 cells were mixed with anti human VNR or FNR antibodies, and the mixture incubated for 30 min at 37°C in a 5% CO_2 incubator. Anti-human VNR antibody strongly inhibited Znα2gp-mediated MC3T3-E1 cell adhesion whereas anti-human FNR antibody did not. Approximately 4 μM anti-human VNR antibody almost completely inhibited the cell adhesion. Furthermore, we confirmed that both receptors were equally expressed on the surface of MC3T3-E1 cells by FACScan.

Effect of Kininogens and Their Derivatives on Znα2gp-Mediated Cell Adhesion

Before carrying out the assay, we treated MC3T3-E1 cells with LK, kf-HK, and α1-TPI (kinin- and f1 · 2-free HK) and α2-TPI (kinin-free LK). After incubation for 30 min at 37°C in a 5% CO_2 incubator, we performed a routine assay as described in Materials and Methods. In the presence of about 0.4 μM of kf-HK, the adhesion of MC3T3-E1 cells was almost completely inhibited (Fig.1). In contrast with kf-HK, LK and both kininogen derivatives (α2- and α1-TPIs) accelerated the MC3T3-E1 cell adhesion from 150 to 180% (Fig.1).

Moreover, the effect of domains 1, 2 and 3 derived from the common heavy chain of both kininogens on Znα2gp-mediated MC3T3-E1 cell adhesion was determined. Domain 3 and heavy chain accelerated with dose dependency (up to 1.5 μM) the cell adhesion from 200 to 230%, domain 2 slightly (up to a maximum of 120%) accelerated it and domain 1 had no effect (Fig.2).

The binding strength of these proteins and three domains to MC3T3-E1 cells was analyzed using FACScan. The order of binding strength to MC3T3-E1 cells was kf-HK > domain 3 > 1 $\geq$ 2 $\geq$ VN $\geq$ Heavy chain.

Effect of Synthetic Peptides on Znα2gp-Mediated MC3T3-E1 Cell Adhesion

To identify the specific amino acid sequence coding the accelerating activity for cell adhesion, we synthesized

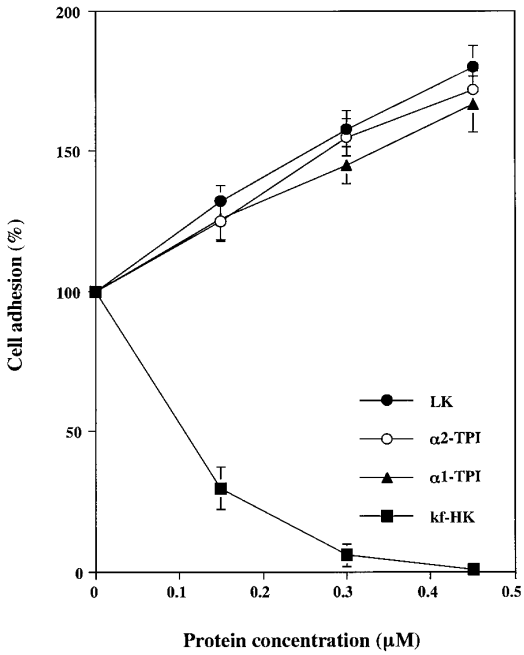

FIG. 1. Effect of kininogens and their derivatives on Znα2gp-mediated cell adhesion. MC3T3-E1 cells (3.5×10^5 cells/ml) were mixed with kininogens and their derivatives and preincubated for 30 min at 37°C. The mixture was added to a 96-well tissue culture plate coated with Znα2gp, and the assay was carried out as described in "Materials and Methods". The data are expressed as a percentage of control at 590 nm, and each point represents the mean ± S.D. of five replicates.

nine peptides covering all amino acid residues of domain 3 (amino acid residue: 235-357) (Fig.3). Among nine peptides (P-1~P-9) and one control peptide (P-8s) which scrambled the amino acid sequence of P-8, peptide-8 (P-8, amino acid sequence: N^{334}AEVYVVPWEKKIYPTVN^{351}) accelerated the Znα2gp-mediated MC3T3-E1 cell adhesion up to approximately 210% (Fig.4). However, P-8s (WENPKVKYVIPYAVENVT) did not affect the cell adhesion. Moreover, the acceleration of MC3T3 E-1 cell adhesion by P-8 was dose-dependent on Znα2gp or VN-coated plate, but not on FN-coated plate whereas P-4 did not accelerate MC3T3-E1 cell adhesion (Fig.5). Under a phase contrast microscopy, MC3T3-E1 cells treated with P-8 attached in approximately 2-fold the number of control and spread with spindle-shaped form (lamellipodia) on Znα2gp-coated plate, but the cells treated with P-8s or with no treatment attached and spread on the plate with membrane-ruffling form (Figure not shown).

The binding strength of P-8 and P-8s to MC3T3-E1 cells was analyzed using FACScan. The binding strength of P-8 to MC3T3-E1 cells was stronger than that of P-8s.

DISCUSSION

We provided evidence that LK and its derivatives as well as HK derivatives regulate Znα2gp-mediated MC3T3-E1 cell adhesion. LK, its derivatives (α2-TPI and heavy chain) and an HK derivative (α1-TPI) accelerated MC3T3-E1 cell adhesion on Znα2gp-coated plate, but kf-HK inhibited the cell adhesion. In fact, among three cystatin-like domains (domains 1-3) comprising the heavy chain, only domain 3 accelerated MC3T3-E1 cell adhesion (Fig.2). The acceleration was almost completely suppressed by the monoclonal antibody against domain 3 (data not shown). Meloni and Schmaier, and Jiang *et al.* (21,20) reported that domain 3 of the LK-heavy chain binds to platelets, and inhibits thrombin-induced platelet activation by preventing thrombin binding to platelets. Reddigari *et al.* (23) reported that the heavy and light chains of HK are capable of directly binding to human umbilical vein endothelial cells (HUVEC). They speculated that the binding between HK and HUVEC results in the contact activation of the initial phase of blood coagulation and kinin formation on the HUVEC cell surface. Furthermore, Herwarld *et al.* (24) indicated that an endothelial binding site in kininogen domain 3 is located in the carboxy-terminal portion and the binding site overlaps the cysteine (thiol) protease inhibitory region. To identify the region possessing the acceleration activity, we synthesized nine peptides covering domain 3. One peptide designated as P-8 accelerated MC3T3-E1 cell adhesion. We observed that P-8 bound to MC3T3 E-1

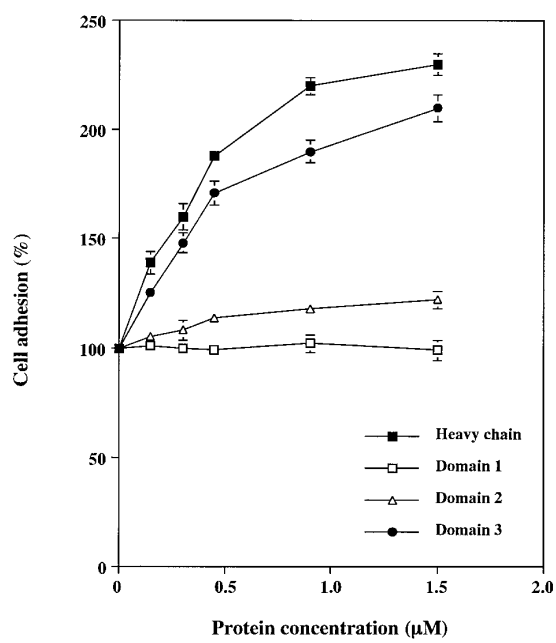

FIG. 2. Effect of heavy chain and three domains (domains 1, 2 and 3) on Znα2gp-mediated cell adhesion. MC3T3-E1 cells (3.5×10^5 cells/ml) were mixed with heavy chain and domains 1–3 and preincubated for 30 min at 37°C. The mixture was added to a 96-well tissue culture plate coated with Znα2gp, and the assay was carried out as described under "Materials and Methods". The data are expressed as a percentage of control at 590 nm, and each point represents the mean ± S.D. of five replicates.

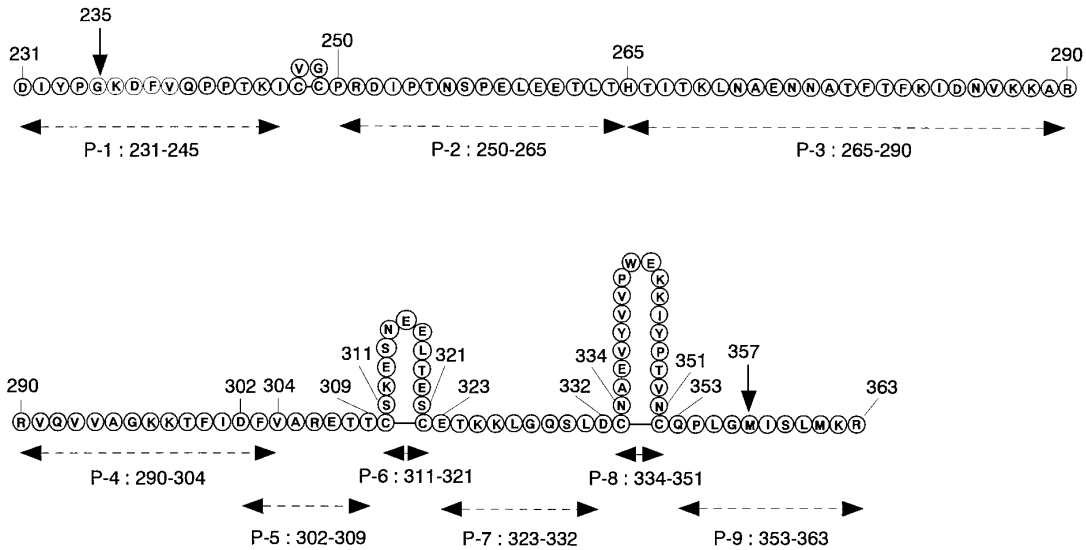

FIG. 3. Synthetic peptides derived from domain 3 of human kininogens. Peptides are designated as P-1∼P-9, followed by the relative amino acid residue numbers of the plasma form of human kininogens.

cells, and competitively interfered with the binding of domain 3 to MC3T3-E1 cells by FACScan (Figure not shown). These findings indicate that the activity of P-8 may be specific for the acceleration of cell adhesion. In addition, the findings of synthetic peptides such as RGDS and ELRGDV and integrins $\alpha5\beta1$ (FNR) and $\alpha v\beta3$ (VNR) antibodies on the MC3T3-E1 cell adhesion strongly suggested that the RGD region of the Znα2gp interacts with integrin $\alpha v\beta3$ molecule on the cell surface of MC3T3-E1 cells. Accordingly, it is speculated that P-8 binds to the integrin-independent receptor(s) on the cell surface and transfers the signal(s) into the inside of the cell, resulting in the acceleration of the cell adhesion on Znα2gp- or VN-coated plate via the expression of integrin $\alpha v\beta3$. However, the mechanism of this signal transduction, so far, is unknown.

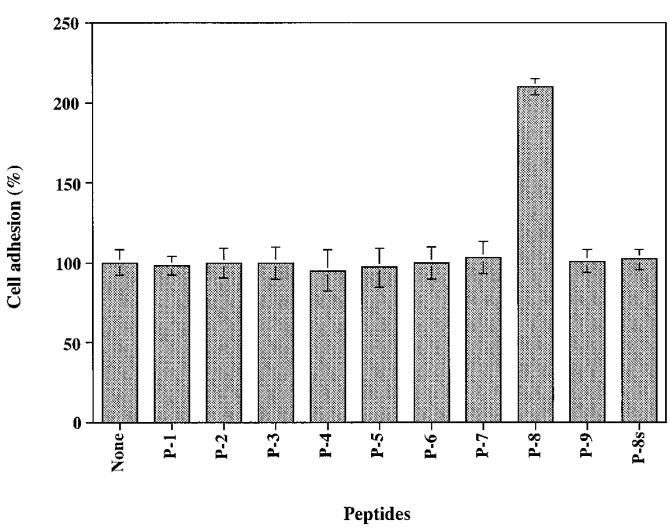

FIG. 4. Effect of synthetic peptides (P-1∼P-9) on Znα2gp-mediated cell adhesion. MC3T3-E1 cells (3.5×10^5 cells/ml) were mixed with 100 μM of P-1∼P-9, and preincubated for 30 min at 37°C. The mixture was added to a 96-well tissue culture plate coated with Znα2gp, and the assay was carried out as described in "Materials and Methods". The data are expressed as a percentage of control at 590 nm, and each point represents the mean ± S.D. of five replicates.

On the other hand, MC3T3-E1 cell spreading on the Znα2gp-coated plate was strongly inhibited by kf-HK. Asakura *et al.* (9) reported that adhesion of the osteosarcoma cell line, MG-63, to vitronectin-coated plate was inhibited by kf-HK. We also confirmed that kf-HK inhibited MC3T3-E1 cell adhesion mediated by Znα2gp. Since the Znα2gp- and VN-coated cell adhesions are mediated through VNR (integrin $\alpha v\beta3$), it is suggested that kf-HK as a disintegrin-like protein directly interfere the function of $\alpha v\beta3$ integrin. The monoclonal antibody against f1 · 2 region of HK neutralized the anti-adhesive activity of kf-HK, but other monoclonal antibodies against other functional domains of HK did not (data not shown). Judging from the datum and the acceleration effect of α1-TPI (kinin- and f1 · 2-free HK), it appears that the f1 · 2 region also plays an important role in the inhibition of cell adhesion. In spite of having the heavy chain, kf-HK does not accelerate MC3T3-E1 cell adhesion. This also indicated that the inhibition activity of the f1 · 2 region is stronger than the acceleration activity of the heavy chain.

As for the pathological and physiological significance(s) of the acceleration and inhibition of cell adhesion, it is speculated that kininogens and their derivatives through processing by proteases such as

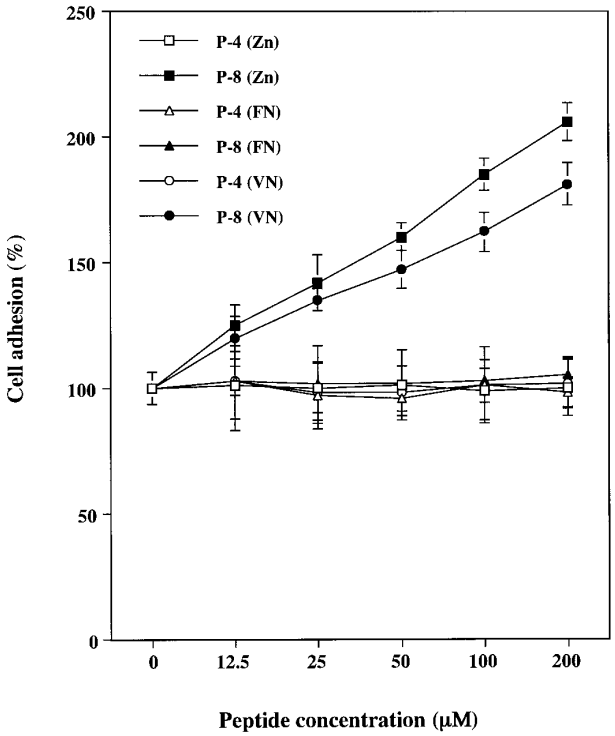

FIG. 5. Effect of synthetic peptides (P-8 and P-4) on Znα2gp-, VN- and FN-mediated MC3T3-E1 cell adhesion. MC3T3-E1 cells (3.5×10^5 cells/ml) were mixed with P-8 or P-4, and preincubated for 30 min at 37°C. The mixture was added to a 96-well tissue culture plate coated with Znα2gp, VN or FN and the assay was carried out as described under "Materials and Methods". The data are expressed as a percentage of control at 590 nm, and each point represents the mean ± S.D. of five replicates.

kallikrein, XIIa, XIa and plasmin (13, 25-27) regulate the adhesion of cells such as osteoblastic cells or osteosarcoma on VN and Znα2gp substrata. As mention above, some investigators indicated that kininogens are capable of binding to platelets, endothelial cells and neutrophils (21-25, 28,29). Based on their and our data, on the early phase of inflammation or blood coagulation cascade, we conclude kinin-free kininogen (kf-HK) formed by the action of XIIa, XIa, kallikrein and plasmin contributes to the inhibition of cell adhesion. When the proteolysis of kininogens is progressed by these serine proteases, kinin- and f1 · 2-free HK (α1-TPI) generated from kf-HK lose their inhibitory activity, and express accelerating activity, contributing to a rapid repair of wounded local tissues. Furthermore, it is also speculated that LK and its derivatives contribute to cell adhesion of osteoblast to extracellular matrix proteins such as VN, Znα2gp and OPN, resulting in local bone formation after bone fracture and inflammation. However, further investigations are needed to comprehend whether this phenomenon is specific in the bone tissue or universal in many tissues. Moreover, it is thought that kininogens and their derivatives may produce contrary reactions in metastasis of cancer cells. If the specific amino acid sequence of the f1 · 2 region was to be identified, we could utilize the synthetic peptide(s) to prevent cancer metastasis. Furthermore, the synthetic peptide, NAEVYVVPWEKKIYPTVN, with promoting activity of cell adhesion in the heavy chain, may be an effective reagent for wound healing in tissue injured by inflammation.

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