Biochimica et Biophysica Acta, 1159 (1992) 215–222

BBAPRO 34302

Heat and autoclave resistance of cell-spreading activity of vitronectin

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(Received 11 March 1992)
(Revised manuscript received 12 May 1992)

Key words: Vitronectin; Cell-spreading; Heat resistance; Autoclave resistance; RGD sequence; Sterilization

We have investigated the heat- and autoclave-resistant properties of the cell-spreading activity of vitronectin, a cell-spreading glycoprotein in animal blood plasma. Vitronectin heated at 100°C for 10 min or autoclaved at 121°C at 1.2 kg/cm^2 for 20 min retained the same cell-spreading activity as native vitronectin. In contrast, fibronectin and type-I collagen treated in the same way lost their activity almost completely. GRGDSP remarkably inhibited the cell-spreading activity of native, heated and autoclaved vitronectins. GRGESP did not inhibit the activity of native vitronectin, but, unexpectedly, partially inhibited the activity of both heated and autoclaved vitronectins. In SDS-polyacrylamide gel analysis under reducing conditions, vitronectin heated at 100°C migrated mainly as a monomer, but autoclaved vitronectin migrated at both the top and front of the gel instead of at the position of the monomer. The change in molecular size during the heat- and autoclave treatments was partially prevented by adding 10 mM dithiothreitol or 2% 2-mercaptoethanol to the protein solution.

Introduction

Vitronectin is an RGD-dependent cell-spreading glycoprotein present in various animals' blood plasma [1,2], platelets [3], atherosclerotic lesions [4] and extracellular matrix [5]. It binds to heparin [6,7], collagen [8,9], thrombin-antithrombin III complex [10], plasminogen activator inhibitor 1 (PAI-1) [11] and membrane attack complex [12]. It plays important regulatory roles in immune complement [12], blood coagulation [13] and fibrinolytic systems [11].

Heparin-binding activity of vitronectin is augmented by treatment with 8 M urea [6,7]. This property led us to establish a novel purification procedure of vitronectin from human blood plasma [14], which enables us to purify decigram quantities of blood vitronectin easily and rapidly from a variety of animals [1,2]. The isolated animal vitronectins are similar in their relative molecular mass, cell-spreading activity, and amino-terminal sequences [1,2]. However, these vitronectins vary in sugar moiety and in the number of polypeptides. Human vitronectin has, for example, only *N*-linked saccharides, while porcine vitronectin has both *N*- and *O*-linked saccharides. Besides, in SDS-PAGE under reducing conditions, human vitronectin is identified as a mixture of two polypeptides of 75 and 65 kDa, while porcine vitronectin is a uniform polypeptide of 59 kDa [1,2]. The human 65-kDa polypeptide is a cleaved form of the 75-kDa polypeptide [15,16].

The successful mass-preparation of vitronectin from human blood seems to provide a possible clinical application of vitronectin similar to fibronectin, a similar cell-spreading glycoprotein in animal plasma. Fibronectin has been used as an eye lotion for corneal epithelial wound healing [17]. However, during the clinical usage of blood proteins, care should be taken for eliminating the possible contaminating microorganisms such as hepatitis and AIDS viruses. Hepatitis and AIDS viruses are reported to be inactivated by heating at 60°C for 10 h and 56°C for 20 min, respectively [18,19]. But, in order to be convenient and sure, the World Health Organization (WHO) recommends boiling for 20 min and autoclaving at 121°C at 1.1 kg/cm^2 for 20 min as the conditions for high-level disinfection and sterilization, respectively [20]. So, it is desirable to examine the heat resistance of cell-spreading glycoproteins.

Generally, animal tissue culture requires fetal bovine serum as one of the supplements to the medium. Fetal bovine serum provides a cell-spreading factor, which

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Abbreviations: RGD, Arg-Gly-Asp; GRGDSP, Gly-Arg-Gly-Asp-Ser-Pro; GRGESP, Gly-Arg-Gly-Glu-Ser-Pro.

has been thought to be fibronectin, but is recently determined to be vitronectin. The cell attachment activity attributable to vitronectin is 8–16-fold greater than that of fibronectin, making vitronectin the main adhesive protein in fetal bovine serum [21]. When cells are plated in medium containing bovine serum stripped of fibronectin, they attach and grow normally, whereas in medium containing serum stripped of vitronectin, cells fail to attach and grow [22]. In routine cell cultures containing bovine serum, integrin at focal contacts is dominated by clusters of vitronectin receptors and not of fibronectin receptors, suggesting that vitronectin and not fibronectin is virtually working as the cell-spreading factor on the substratum [23]. Therefore, easy sterilization for vitronectin appears to promote further development of serum-free cell culture.

Here, we focus on the nature of the cell-spreading activity of vitronectin, and investigate the effect of heat and autoclave treatment on the activity. In this paper, we report that the cell-spreading activity of vitronectin is heat-resistant and even autoclave-resistant, while that of fibronectin is not. Furthermore, the cell-spreading of heated and autoclaved vitronectins is shown to mediate an RGD sequence.

Experimental Procedures

Cell-adhesive proteins

Vitronectin was purified from human and porcine plasma using heparin-affinity chromatography as a heparin-binding protein in the presence of 8 M urea according to Yatohgo et al. [14]. Barnes-type vitronectin, which is a heparin-unbinding form of vitronectin, was also isolated from human plasma in the absence of 8 M urea according to Barnes and Silnutzer [24]. Fibronectin was prepared by gelatin-affinity chromatography from human plasma as previously described [25]. Laminin was isolated from mouse EHS sarcoma according to Timpl et al. [26]. Type-I collagen from porcine skin was purchased from Nitta Gelatin (Osaka, Japan). To examine the effect of 8 M urea, fibronectin (0.25 mg/ml) was treated with 8 M urea, 0.13 M NaCl and 10 mM sodium phosphate (pH 7.2) for 2 h at room temperature and then dialyzed against 0.13 M NaCl and 10 mM sodium phosphate (pH 7.2) at room temperature. The dialysate was used as urea-treated fibronectin.

Preparation of heated and autoclaved proteins

Proteins were diluted with 0.13 M NaCl and 10 mM sodium phosphate (pH 7.2) at the concentration of 0.25 mg/ml. The diluted protein solution (100 μl) in glass vials or polypropylene microtubes was incubated at the indicated temperature in a water bath for 10 or 60 min or was autoclaved at 121°C at 1.2 kg/cm^2 for 20 min. Care was taken to reduce evaporative loss using loose caps. Protein recovery after these treatments was confirmed by absorbance measurements at 280 nm. The recovery of autoclaved vitronectin was also determined according to Lowry et al. [27]. The effect of reducing agents was examined by adding dithiothreitol (Bethesda Research Laboratories) or 2-mercaptoethanol (Nacalai Tesque, Kyoto, Japan) to the protein solution at the final concentration of 10 mM or 2%, respectively, just prior to treatment.

SDS-PAGE

SDS-PAGE was essentially performed according to the method of Laemmli [28] using 10% acrylamide for the separation gels and 4% acrylamide for the stacking gels. The samples were treated with a sample buffer of 10 mM sodium phosphate (pH 6.8), 2% SDS, 10% glycerol, 0.003% Bromophenol blue, 0.4 mM phenylmethylsulfonyl fluoride and 5% 2-mercaptoethanol. They were incubated in a boiling water bath for 5 min and subjected to SDS-PAGE. Protein bands were visualized by staining with 0.25% Coomassie blue followed by destaining with 5% methanol and 7.5% acetic acid. The recovery of autoclaved vitronectin after the treatment with sample buffer was confirmed as follows: Autoclaved vitronectin was incubated with sample buffer in a boiling bath for 5 min and then dialyzed against 0.13 M NaCl and 10 mM sodium phosphate (pH 7.2) at 4°C. The protein concentration of the dialysate was determined by the method of Lowry et al. [27].

Cell-spreading assay

The cell-spreading assay was carried out essentially as described previously [29]. BHK fibroblastic cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were harvested with 0.25% trypsin, collected by centrifugation and suspended in Grinnell's adhesion medium (150 mM NaCl, 1 mM $CaCl_2$, 3 mM KCl, 0.5 mM $MgCl_2$, 6 mM Na_2HPO_4, and 1 mM KH_2PO_4, pH 7.3) [30] containing 0.35% soybean trypsin inhibitor. After being centrifuged again, the cells were resuspended in Grinnell's adhesion medium. Polyvinyl chloride microtiter plates with 96 wells (ELISA E, Sumitomo Bakelite, Tokyo, Japan) were coated with various concentrations of heated, autoclaved or intact proteins in the adhesion medium at 37°C for 1 h. Unbound proteins were removed by aspiration. Cells were added to the well ($1 \cdot 10^4$ cells in 0.1 ml/well) and incubated at 37°C for 90 min. Cells were fixed with phosphate-buffered saline containing 2% glutaraldehyde, 5% formaldehyde and 5% sucrose at room temperature for 20 min. The cell-spreading activity was presented as the percentage of cells spread per cells attached after counting under a microscope. The effect of RGD-containing peptides on the cell-spreading of

vitronectin was examined by adding synthetic peptides (100 μg/ml) to cell suspension on the well coated with 10 μg/ml vitronectin.

Results

Cell-spreading activity of heated and autoclaved proteins

Cell-spreading activity of human vitronectin, porcine vitronectin, human fibronectin, porcine type-I collagen and mouse laminin was tested. As shown in Fig. 1, BHK cells spread on human vitronectin, human fibronectin and porcine type-I collagen in a dose-dependent manner, but failed to spread on mouse laminin. Porcine vitronectin was also active in the cell-spreading of BHK cells in the same manner as human vitronectin (data not shown). Both human and porcine vitronectins promoted the spreading of BHK cells more efficiently than fibronectin and type-I collagen.

The susceptibility of the cell-spreading activity to heat and autoclave treatments was examined. Human vitronectin, porcine vitronectin, human fibronectin and porcine type-I collagen were heated at various temperatures ranging from 40 to 100°C for 10 min or autoclaved at 121°C at 1.2 kg/cm^2 for 20 min. Surprisingly, the cell-spreading activity of human vitronectin was retained almost entirely after the heat treatment and even after autoclaving (Fig. 2). Porcine vitronectin also had the same heat-resistant cell-spreading activity as human vitronectin. In marked contrast, the activity of

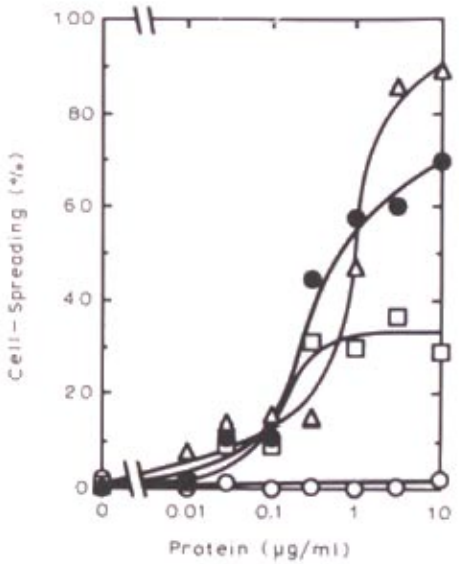

Fig. 1. Cell-spreading of BHK cells on glycoproteins. BHK cells suspended in Grinnell's adhesion medium were allowed to spread on polyvinyl chloride microtiter wells which had been previously coated with human vitronectin (●), human fibronectin (△), porcine type-I collagen (□), and mouse laminin (○) at the indicated concentrations (see Experimental Procedures for details). After 90 min at 37°C, cells were fixed and counted under a phase-contrast optical microscope. Cell-spreading activity is expressed as the percentage of cells spread of the total number of cells attached.

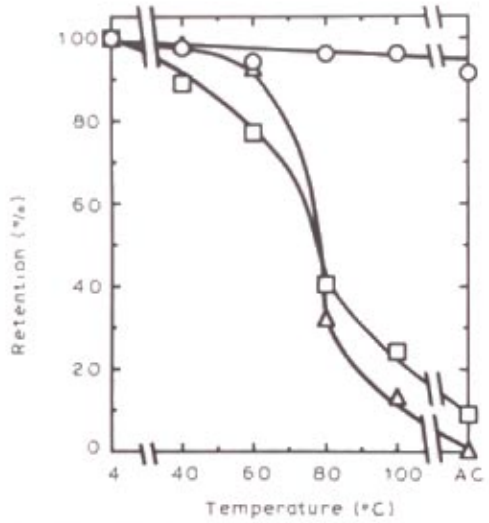

Fig. 2. The retention of the cell-spreading activity of heated and autoclaved proteins. Human vitronectin (○), human fibronectin (△), and porcine type-I collagen (□) were incubated at the indicated temperature for 10 min or autoclaved at 121°C at 1.2 kg/cm^2 for 20 min (indicated as AC) at a concentration of 0.25 mg/ml in 0.13 M NaCl and 10 mM sodium phosphate buffer (pH 7.2). Microtiter wells were coated with heated or autoclaved proteins of 10 μg/ml in Grinnell's adhesion medium at 37°C for 60 min. The cell-spreading assay was carried out using BHK cell suspension at 37°C for 90 min. The retention of the activity is expressed as the percentage of the cell-spreading activity retained after heat and autoclave treatments compared before the treatments.

fibronectin and type-I collagen decreased in a temperature-dependent manner and finally almost disappeared after autoclaving. The activity of vitronectin was still resistant to longer incubation times up to 60 min at 100°C. With respect to specific activity, both heated and autoclaved vitronectins were similar to native vitronectin (Fig. 3).

The morphology of BHK cells spread on vitronectin heated at 100°C (Fig. 4B) or autoclaved (Fig. 4C) was similar to that on native vitronectin (Fig. 4A) and fibronectin (Fig. 4D). However, the cells did not spread on fibronectin heated at 100°C (Fig. 4E) or autoclaved (Fig. 4F). These results indicate that the cell-spreading activity of vitronectin was resistant to heat treatment at 100°C for 10 min and autoclaving at 121°C at 1.2 kg/cm^2 for 20 min, but that of fibronectin and type-I collagen was not.

We examined whether the heat and autoclave resistant cell-spreading activity of vitronectin was due to the effect of urea treatment of protein during purification. Barnes-type vitronectin, which is a heparin-unbinding form of vitronectin and not treated with 8 M urea during purification, also showed the same heat and autoclave resistance in the cell-spreading activity (data not shown). Fibronectin, even treated with 8 M urea, however, lost the cell-spreading activity after heating at 100°C for 10 min or autoclaved at 121°C at 1.2 kg/cm^2

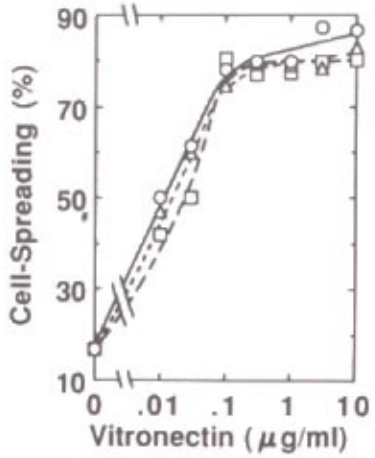

Fig. 3. Dose response of the cell-spreading activity of intact, heated and autoclaved human vitronectins. Intact vitronectin (○, ———) was heated at 100°C for 10 min (△, ·····) or autoclaved at 121°C at 1.2 kg/cm² for 20 min (□, — — —) and was allowed to coat microtiter wells at the indicated concentrations. The cell-spreading assay was carried out using BHK cell suspension at 37°C for 90 min.

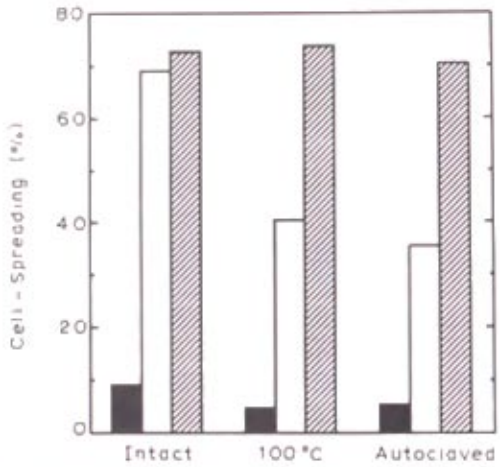

Fig. 5. Inhibition of cell-spreading by synthetic peptides. Microtiter wells were coated with human intact vitronectin, vitronectin heated at 100°C for 10 min, or vitronectin autoclaved at 121°C at 1.2 kg/cm² for 20 min at 10 μg/ml. BHK cells were allowed to spread on the coated-wells in the presence of 100 μg/ml of GRGDSP (closed bar) or GRGESP (open bar). Shaded bar represented the cell-spreading activity in the absence of peptide as a control.

for 20 min. These results indicate that the heat and autoclave resistance of cell-spreading activity is intrinsic to vitronectin and is not created by the treatment with 8 M urea.

Inhibition of the cell-spreading activity of heated and autoclaved vitronectins by RGD-containing peptides

Although the cell-spreading on vitronectin and fibronectin is generally known to mediate a common RGD sequence within both molecules [31,32], the cell-spreading activity of vitronectin was resistant to heating and autoclaving, but that of fibronectin was not. This difference raised the question as to whether the RGD site was still responsible for the cell-spreading activity of heated and autoclaved vitronectins. If it is responsible, the activity is inhibited by RGD-containing peptides [33]. The cell-spreading of these vitronectins was examined in the presence of 100 μg/ml GRGDSP or GRGESP. The GRGDSP peptide almost completely inhibited the cell-spreading on both heated and native vitronectins (Fig. 5). These results suggest that the primary cell-binding site of heated and autoclaved vitronectins was the same RGD sequence as that of native vitronectin and that the RGD-dependent cell-spreading of vitronectin was resistant to heating and to autoclaving but that of fibronectin was not. However, the response to the GRGESP peptide was

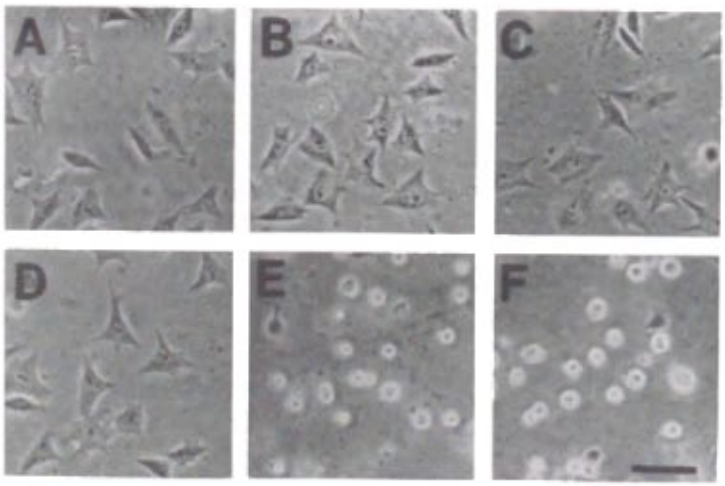

Fig. 4. The morphology of BHK cells on human vitronectin (A–C) or human fibronectin (D–F). Proteins were heated at 100°C for 10 min (B and E) or autoclaved at 121°C at 1.2 kg/cm² for 20 min (C and F) and were allowed to coat microtiter wells at 10 μg/ml. Intact proteins without the treatment were also allowed to coat microtiter wells as a control (A and D). BHK cells were incubated on the coated wells at 37°C for 90 min. The morphology was observed under a phase-contrast optical microscope. Bar, 50 μm.

different among native, heated and autoclaved vitronectins (Fig. 5). The GRGESP peptide did not inhibit the cell-spreading activity of native vitronectin, but did inhibit the activity of both heated and autoclaved vitronectins by approx. 50%.

SDS-PAGE of heated and autoclaved vitronectins

To determine whether the vitronectin molecule maintains its intact molecular size after the heat and autoclave treatments, we compared the electrophoretic profile of heated and autoclaved vitronectins by SDS-PAGE under reducing conditions. Both human (Fig. 6, lane 2) and porcine (Fig. 6, lane 5) vitronectins heated at 100°C for 10 min mainly migrated the same as those of non-heated vitronectins having relative molecular masses of 75, 65 (Fig. 6, lane 1) and 59 kDa (Fig. 6, lane 4), respectively. A minor difference was found in the appearance of the high molecular mass materials of more than 120 kDa, which may be vitronectin multimers. The size change was more conspicuous at 100°C after 60 min than after 10 min (Fig. 7, lane 2). The

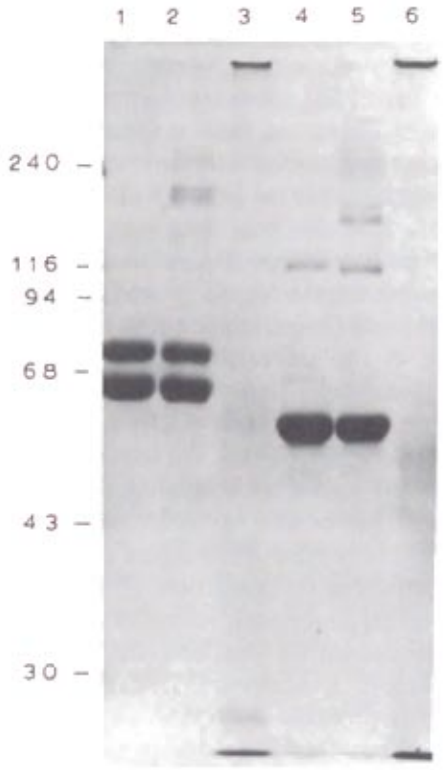

Fig. 6. SDS-PAGE of heated and autoclaved vitronectins. Human (lanes 1–3) and porcine vitronectins (lanes 4–6) were heated at 100°C for 10 min (lanes 2 and 5) or autoclaved at 121°C at 1.2 kg/cm^2 for 20 min (lanes 3 and 6). The heated and autoclaved vitronectins were treated with sample buffer containing 5% 2-mercaptoethanol in a boiling water bath for 5 min. They were run on SDS-PAGE consisting of 10% acrylamide for a separation gel and 4% acrylamide for a stacking gel. Controls were intact vitronectin (lanes 1 and 4). 7.5 μg of vitronectin was applied in each lane. The numbers indicated on the left denote the relative molecular mass of the standard proteins in kDa

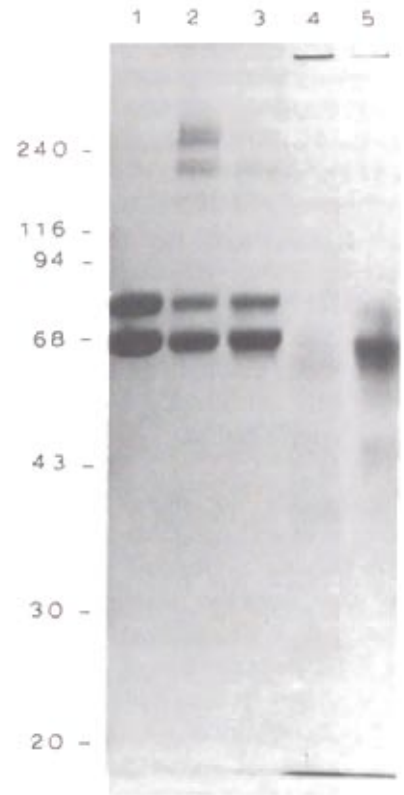

Fig. 7. Effect of dithiothreitol on the change in relative molecular mass during heat and autoclave treatments. Human vitronectin (lane 1) was incubated at 100°C for 60 min (lanes 2 and 3) or autoclaved at 121°C at 1.2 kg/cm^2 for 20 min (lanes 4 and 5) in the presence (lanes 3 and 5) or absence (lanes 2 and 4) of 10 mM dithiothreitol. Vitronectin was run on SDS-PAGE under reducing conditions. The numbers indicated on the left denote the relative molecular mass of the standard proteins in kDa. 7 μg of vitronectin was applied in each lane.

recovery of vitronectin after heating at 100°C for 10 min was estimated to be 98% from the absorbances at 280 nm.

The profiles of autoclaved vitronectins (Fig. 6, lanes 3 and 6) were very different from those of native and heated vitronectins. Protein bands at around 75, 65 and 59 kDa almost disappeared. Instead, two extraordinary bands appeared a very large molecular weight band which did not enter into the stacking gel of 4% acrylamide and a very small band which migrated at the front of the 10% acrylamide separation gel, corresponding to a relative molecular mass of less than 20 kDa. The very large molecular weight material was not sedimented by centrifugation at 9000 $\times g$ for 5 min. It filtered through a polytetrafluoroethylene filter with a pore size of 0.45 μm. To exclude the possibility that the disappearance of 75 and 65 kDa bands in autoclaved vitronectin was due to the loss of vitronectin during autoclaving and treatment with sample buffer, protein recovery after these processes was confirmed. The value of vitronectin in 0.13 M NaCl and 10 mM

sodium phosphate (pH 7.2) monitored at 280 nm changed from 0.308 to 0.414 after the autoclaving step, but the absorbance of the solvent also changed from 0.006 to 0.126. Thus, the recovery of vitronectin was calculated to be 95.4%. Furthermore, the protein determination by the method of Lowry et al. [27] resulted in vitronectin recovery of 95.2%, which was consistent with the value determined by the absorbance at 280 nm described above. After treatment with the electrophoresis sample buffer, autoclaved vitronectin was recovered at 91.5%. Therefore, the disappearance of 75 and 65 kDa bands was not due to the low recovery of vitronectin after autoclaving and treatment with electrophoresis sample buffer.

Prevention against the size change of the vitronectin molecule during heat and autoclave treatments

Vitronectin became aggregated and degraded during the heat and autoclave treatments as described above. To prevent a size change, we added dithiothreitol at a final concentration of 10 mM during the treatments. As shown in Fig. 7, dithiothreitol was remarkably, but not completely, effective at both heating at 100°C (Fig. 7, lane 3) and autoclaving (Fig. 7, lane 5). This prevention effect was also observed with the addition of 2% 2-mercaptoethanol instead of dithiothreitol, suggesting that the size change of vitronectin was partially due to oxidation during the heat and autoclave treatments. The vitronectin heated or autoclaved in the presence of dithiothreitol had the same cell-spreading activity as native vitronectin (data not shown).

Discussion

In this study, we have investigated the heat and autoclave resistance of the cell-spreading activity of vitronectin. Vitronectin heated at 100°C for 10 min or autoclaved at 121°C at 1.2 kg/cm^2 for 20 min retained the same cell-spreading activity as non-heated vitronectin, which represented half-maximal activity at 0.03 μg/ml. The heat and autoclave resistance was similar between human and porcine vitronectins, indicating that the heat resistance seems to be common to animal vitronectins. Since the sugar composition is different between human and porcine vitronectins [2], the heat and autoclave resistance would not depend on the sugar moiety in vitronectin. Human vitronectin is composed of two bands of 75 and 65 kDa, while porcine vitronectin has only one band of 59 kDa. Also, the heat and autoclave resistance would not depend on band number of one or two.

Barnes-type vitronectin, which is isolated in the absence of 8 M urea as a heparin-unbinding protein, also has heat and autoclave resistant cell-spreading activity. Moreover, fibronectin was sensitive to heating and autoclaving in the cell-spreading activity even after treatment with 8 M urea. Thus, the heat and autoclave resistance of cell-spreading activity of vitronectin is independent of the property of heparin-binding activity and the treatment with 8 M urea.

Goodman et al. [34] reported that 20–40% of the cell-attachment activity of laminin is heat stable. Rat glioblastoma cells attach and spread on laminin or laminin fragments of P1 and E1-X. The attachment activity of laminin decreases between 60 and 65°C to 20–40%, which is, however, retained after heat treatment up to 100°C. Furthermore, the cell-attachment activity of P1 and E1-X, which occurs in an RGD-dependent manner, is not affected by the heat treatment between 0 and 100°C. In our experiments, the cell-spreading activity of the whole vitronectin molecule was stable over a wide range of temperatures and never decreased. We did not observe the heat-resistant activity of laminin, because BHK cells did not spread on laminin (Fig. 1).

Since the cell-spreading activity of both heated and autoclaved vitronectins was almost completely inhibited by an RGD-containing peptide, the activity of heated and autoclaved vitronectins also appears to mediate the RGD sequence in common with intact vitronectin. There has been no report on the second cell-binding site in vitronectin in contrast to fibronectin and other cell-adhesion proteins [34–37]. Therefore, it is unlikely that the heat and autoclave resistance of vitronectin depends on the second cell-binding site, which is resistant to heating and autoclaving. Both vitronectin and fibronectin seem to use the same RGD sequence in the primary structure to generate cell-spreading activity. There are, however, specific cell surface receptors. Integrin $\alpha_5\beta_1$, for example, recognizes fibronectin but not vitronectin, while integrin $\alpha_v\beta_3$ has the opposite recognition [38]. Pierschbacher et al. [39] reported that the conformation of the RGD sequence is important for receptor recognition. Therefore, a different conformation of RGD between fibronectin and vitronectin seems to exist, which seems to be one reason for the different sensitivity to heat and autoclave treatments. GRGDSP is a sequence found in fibronectin, but does not quite exist in vitronectin. When used as an affinity ligand, the GRGDSP peptide binds to integrin $\alpha_v\beta_3$ but not to integrin $\alpha_5\beta_1$ [38]. Conformation of the RGD sequence in the synthetic peptide, an obviously heat-resistant peptide, is interpreted to resemble that in native vitronectin. This is in good agreement with the heat and autoclave resistant activity of vitronectin. In contrast to this interpretation, Vogel et al. [40] recently reported that integrin $\alpha_v\beta_1$ binds to both fibronectin and GRGDSP, but not to vitronectin, suggesting a different conformation of the RGD sequence between the synthetic peptide and native vitronectin. We assume that the heat and

autoclave resistance should be due to the heat-resistant conformation of the RGD sequence in vitronectin. The conformation of the RGD sequence in fibronectin should be heat labile.

The conformation around the RGD sequence of vitronectin, however, seems to be partially changed by heat and autoclave treatments, because a GRGESP peptide, a negative control peptide, partly inhibited the cell-spreading activity of both heated and autoclaved vitronectins but not the native vitronectin. The conformation around the RGD sequence in both heated and autoclaved vitronectins seems to compete with the GRGESP peptide in terms of affinity to cell surface integrin. Recently, Brake et al. [41] reported that the human immunodeficiency virus-1 transactivation protein, tat, whose RGD sequence is replaced into RGE by mutation, slightly promotes the cell adhesion of monocytic and T-lymphocytic cells, as does the wild-type of tat. Mould et al. [42] reported that a GRGES peptide inhibits the adhesion of melanoma cells to CS-1 and CS-5 peptides as well as RGD-containing peptides. CS-1 and CS-5 are synthetic fibronectin peptides with cell-binding sequences of LDV and REDV, respectively, for melanoma cells but not for BHK cells [43,44]. Our results suggest that the RGE sequence can also be weakly recognized by BHK cells.

The molecular size of fibronectin is three times larger than that of vitronectin. In addition, the RGD site of fibronectin is located in the middle of the molecule, whereas that of vitronectin is near the amino-terminus. Although we have discussed that the resistance seems to depend on the conformation of the RGD sequence in adhesion proteins, we cannot exclude the possibility that the RGD site is spatially hindered by heat and autoclave treatments in fibronectin, but not in vitronectin.

Heating of protein at 100°C can cause peptide-bond cleavages, which occur predominantly at an aspartyl–prolyl peptide bond [45,46]. Cleavage and precipitation of β-galactosidase by autoclaving at 121°C has been observed [47]. Vitronectin was also cleaved and aggregated slightly by heating at 100°C for 10 min and very significantly by autoclaving at 121°C at 1.2 kg/cm^2 for 20 min. Human vitronectin has two aspartyl–prolyl peptide bonds in the molecule, which correspond to residue Asp-217–Pro-218 and Asp-439–Pro-440 [48]. These bonds seem to be cleaved preferentially by heating and autoclaving. Vitronectin also formed multimers forms and aggregated. Since cleavage and aggregation by heat and autoclave treatments have been partially inhibited by the addition of 10 mM dithiothreitol or 2% 2-mercaptoethanol (Fig. 7), it appears that the oxidation could be, at least partially, a cause for the cleavage and aggregation of vitronectin.

Biological activity of heated and autoclaved vitronectins other than cell-spreading, such as regulatory activity in blood coagulation, fibrinolysis, and the immune complementary system, has not yet been examined. The effect of heat and autoclave treatments on the property of vitronectin, such as the binding to heparin and to collagen and the phosphorylation of vitronectin, was not fully tested either. Heparin-binding activity of vitronectin was activated by 8 M urea [6,7], 6 M guanidine-HCl or heating at 100°C [6]. Endogenous serum vitronectin acquires the binding activity to collagen after heating in the presence of urea and heparin [9]. Thus, the heated and autoclaved vitronectins seem to share some common property with intact vitronectin and keep some biological activity.

The heat and autoclave resistance of the cell-spreading activity of vitronectin would enable the easy processing of vitronectin as a biomaterial. The processing conditions used in the field of synthetic polymer engineering are very harsh for proteins, such as high temperature, strong catalyzers and acidic or alkaline pH. The cell-spreading activity of vitronectin is resistant not only to heating and autoclaving, but also to treatment at pH 2.5 (Nagano, Y. et al., data not shown) or pH 9.5 [49], 0.1% SDS at 100°C [50] and 8 M urea at 100°C [14]. Thus, we are able to acquire the active vitronectin in cell-spreading, even through the treatments with heating, autoclaving, high or low pH, urea and SDS. The effect of the other harsh conditions on the cell-spreading activity of vitronectin, such as the treatments with organic and chaotropic solutions, remains to be examined.

Although heat and autoclaving treatment is generally useful as a disinfection and a sterilizing procedure for glass and metals, most proteins, including plasma proteins, are easily denatured and lose their biological activity after heat and autoclave treatments. We could hardly sterilize and disinfect proteins by autoclaving or heating with keeping their biological activity. However, vitronectin is an exception. Vitronectin disinfected and sterilized by autoclaving or heating is fully active in cell-spreading. Vitronectin sterilized by autoclaving seems to be useful as a cell-spreading factor for serum-free tissue culture medium. When administrating serum proteins to patients clinically, one of the major obstacles is infection by virus, such as AIDS and hepatitis viruses which can survive in the protein solutions. In case of fibronectin eye lotion, the patients' own blood plasma is used as a source for fibronectin to avoid the possibility of a blood transmitted disease [17]. However, vitronectin prepared from pooled human plasma is now found to be disinfected or sterilized by heating or autoclaving in this paper according to the WHO guideline. It can be safe and convenient for clinical administration.

Although the autoclaved vitronectin retained the cell-spreading activity as well as vitronectin heated at 100°C, the molecular size drastically changed. The

change was not fully suppressed, even in the presence of reducing agents. From these results, we recommend heating at 100°C for 20 min without reducing agents, rather than autoclaving, to disinfect the vitronectin solution.

Acknowledgements

This work was supported in part by the Hayashi Memorial Foundation for Female Natural Scientist, by a research grant from the Ministry of Education, Science and Culture of Japan and by the Special Coordination Funds of the Science and Technology Agency of the Japanese Government. We thank Masako Izumi and Naomi Nakashima for their preliminary experiments.

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