ENHANCED BIOLOGICAL ACTIVITY OF POLYMERIC OSTEOPONTIN

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Abstract

Osteopontin is a multifunctional glycoprotein with roles in immunomodulation, inflammatory response, tissue mineralization, and tissue remodeling, which are mediated primarily through integrins. Transglutaminase 2 selectively cross-links proteins by isopeptide bonding. Osteopontin is one of the substrates of this enzyme and undergoes polymerization; however, the biological meaning of this polymerization remains unknown. Using recombinant osteopontin polymerized with purified transglutaminase 2, we examined cell adhesion, spreading, focal contact formation, and migration of SW480 or HUVE cells. All of these cellular behaviors were dramatically enhanced with polymeric osteopontin. These enhancements of cellular functions imply that polymerization might modulate physiological and pathological functions of osteopontin.

Key words: Osteopontin; Transglutaminase 2; Integrin; Polymerization

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's Modified Eagle Medium; PBS, phosphate buffered saline; FBS, fetal bovine serum

Introduction

Osteopontin is an acidic phosphorylated glycoprotein with versatile functions, including roles in tissue remodeling, fibrosis, mineralization, immunomodulation, and inflammation [1,2]. The principal ways osteopontin affects cellular behavior is through interactions with integrins or CD44 [3]. Integrins are heterodimeric cell surface glycoproteins that mediate cell response to extracellular matrix proteins [4,5]. Osteopontin contains the canonical integrin recognition sequence, arginine-glycine-aspartic acid (RGD), which is recognized by 6 integrins [2,6]. Two other integrins bind to a non-RGD (SVVYGLR) sequence [7,8]. Transglutaminase 2 (EC 2.3.2.13) is a widely distributed intra- and extracellular calcium-dependent enzyme that catalyzes polymerization of its substrate proteins by creating isopeptide cross-links between glutamine and lysine residues [9]. Osteopontin serves as one of the substrates for this enzyme [10,11]. However, functions of resultant polymeric osteopontin are still unknown except changes in conformation with increased collagen-binding properties [12]. Recently polymeric osteopontin was found *in vivo* in rat bone [11] and in calcified arteries of matrix Gla protein deficient mice [13]. These presences of polymeric osteopontin suggest roles in the normal and pathological tissues. Here, we demonstrated functional significance of the polymerization. Integrin-mediated cell adhesion, spreading and migration were all enhanced.

Materials and Methods

Cells, antibodies, and reagents - The human colon carcinoma cell line SW480 was obtained from American Type Culture Collection. Human umbilical vein endothelial (HUVE) cells were purchased from Cambrex (Walkersville, MD). SW480 cells were used for cell adhesion and migration assays because we have previously characterized the cells for adhesion to several forms of osteopontin. HUVEC cells, which readily extend cytoplasm on a substrate, were used for observation of cell spreading and focal contact formation. Anti-human integrin monoclonal antibodies, anti-α1 (FB12), α2 (P1E6), α4 (P1H4), α5β1 (JBS5), α6 (NKI-GoH3), αvβ3 (LM609), and β1 (P5D2) were purchased from Chemicon (Temecula, CA); anti-α3 (P1B5) and RGD peptide were purchased from Invitrogen (Grand Island, NY). An antibody against αvβ5 (P1F6) was from Dr. Dean Sheppard (UCSF, San Francisco, CA). Anti-human osteopontin polyclonal antibody was obtained from IBL (Takasaki, Japan). Anti-CD151 monoclonal antibody, 8C3, was from Dr. Kiyotoshi Sekiguchi (Osaka University, Osaka, Japan). Fluorescent or HRP conjugated secondary antibodies were from BD Pharmingen (San Diego, CA), Zymed (San Francisco, CA) and Invitrogen. Mouse anti-human phosphotyrosine monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Transglutaminase 2 was purified and provided by Dr. Yuji Saito (Tokyo Institute of Technology, Yokohama, Japan) [14]. Recombinant human osteopontin with or without the RAA mutation was produced as previously described [6,7].

Polymerization of osteopontin by transglutaminase 2 - Recombinant human osteopontin (50 μg/ml) was incubated with transglutaminase 2 at concentrations of 0–200 μg/ml in the reaction buffer [5 mM $CaCl_2$, 1 mM DTT, and 50 mM Tris-HCl (pH 7.5)] at 37 ºC for 2 h. The resultant osteopontin was confirmed by western blotting to have formed a polymer. Polymerized osteopontin

generated with 100 μg/ml transglutaminase 2 was used in the following experiments. Transglutaminase 2 was not removed from the polymerized osteopontin preparation.

Cell adhesion and spreading assay - The cell adhesion assay was performed essentially as described [15]. Flat-bottom 96-well microtiter plates (Nunc Inc., Naperville, IL) were coated with 100 μl of monomeric or polymeric osteopontin in PBS at 4ºC overnight. After wells were blocked with 1% BSA in DMEM, a total of 5×10^4 cells in 100 μl of DMEM were added to each well then incubated for 30 min at 37ºC. The adhered cells were fixed and stained with 0.5% crystal violet for quantification. The optical density of cells adhered to 1% BSA was subtracted in each experiment as background. When blocking antibody was used (0.1 mg/ml), cells were pre-incubated on ice for 15 min. The spreading assay with HUVE cells was performed using the plates coated as described above and assessed by counting cells with extended and transparent cytoplasm after incubation for 30 min at 37ºC.

Cell migration assay - The bottom sides of polycarbonate membranes with 8-μm pores (Transwell, Corning, NY) were coated with 15 μl of 10 μg/ml monomeric or polymeric osteopontin, or 20 μg/ml of transglutaminase 2. 1×10^5 cells were added to the top of each chamber, then allowed to migrate to the lower chambers containing DMEM with 1% FBS for 24 h at 37ºC in a humidified atmosphere with 5% CO_2. Migrated cells on the bottom side of the membrane were stained with crystal violet for counting.

Confocal microscopy - Chamber slides (BD, Franklin Lakes, NJ) were coated with 1.0 μg/ml of monomeric or polymeric osteopontin overnight at 4 ºC. 1×10^5 cells were added to each chamber

and incubated for 30 min at 37 ºC. Cells were fixed and permeabilized, then anti-human phosphotyrosine mouse monoclonal antibody was added and incubated for 1 h followed by incubation with Alexa Fluor 555 anti-mouse IgG. Actin filaments were stained with Alexa Fluor 488 phalloidin. Confocal microscopy analyses were performed with a LSM5 Pascal-V3.2 (Carl Zeiss Japan, Tokyo, Japan).

Results

Transglutaminase 2 catalyzes osteopontin to form polymer - Immunoblotting with the anti-osteopontin polyclonal antibody showed dose-dependent multimerization from 10 to 200 μg/ml of transglutaminase 2, as manifested by the increase in the smeared band with a molecular mass over 200 kDa and a decrease in intensity of single bands less than 30 kDa corresponding to monomeric recombinant osteopontin (Fig. 1A), of which putative molecular weight was 34 kDa. Because intensity of the smeared bands of polymeric osteopontin was much higher than a single band of monomeric osteopontin, amounts of proteins appeared to be different among lanes. However, the same amount of proteins were loaded on each lane. Fig. 1B shows that the purified transglutaminase 2 was not immunoreactive at all with anti-osteopontin antibody.

Enhanced cell adhesion to polymeric osteopontin (Fig. 2). - SW480 cells adhered to either monomeric or polymeric osteopontin in a dose-dependent manner. However, adhesion to polymeric osteopontin was enhanced at all concentrations from 0.1 to 1.5 μg/ml. SW480 cells minimally adhered to transglutaminase 2.

Coating efficiency of monomeric and polymeric osteopontin - The coating solutions were subjected to western blotting after the incubation to visualize unbound osteopontin (Fig. 3A). By densitometry [16] of the blot, monomeric and polymeric osteopontin decreased by about 78 % and 72 %, respectively, presumably reflecting adsorption of the protein to the wells (Fig. 3B). The enhanced adhesion of SW480 cells to polymerized osteopontin was not due to difference in coating efficiency.

Cell adhesion to polymeric osteopontin is mediated by integrins - The adhesion to monomeric osteopontin was completely abrogated by a combination of anti-α5β1 and anti-αvβ5 (Fig. 4A) as previously reported [6]. However, the enhanced adhesion to polymeric osteopontin was only partially blocked (Fig. 4B). We next incubated the cells with GRGDSP peptide or used OPN-RAA in which the RGD sequence was mutated to RAA (OPN-RAA). The peptide treatment completely abrogated cell adhesion of SW480 to monomeric osteopontin but incompletely for polymeric osteopontin. SW480 cells did not adhere to monomeric OPN-RAA but did to the polymeric OPN-RAA. These RGD independent adhesions to polymeric osteopontin, however, were completely abrogated by combination of anti-αvβ5 and β1 blocking antibodies.

Integrin α3β1 is a receptor for polymeric osteopontin - As presented in Fig. 5A, the integrin subunits α1, α2, α3, α5, and α6 were expressed on SW480 cells. Therefore, with a combination of antibodies against these subunits along with anti-αvβ5 and α5β1, we examined which β1-integrin(s) might be implicated in the adhesion. Anti-α3 substantially inhibited this residual adhesion, whereas anti-α1, α2 and α6 had little or no effects (Fig. 5B). Further, combinations

including anti-α3 inhibited residual adhesion, whereas anti-α1, -α2 or α6 made little or no contribution (Fig 5C). A monoclonal antibody against tetraspanin CD151 can inhibit the function of α3β1 by disrupting a complex of α3β1 with CD151 [17]. Addition of the anti-CD151 completely blocked the adhesion to polymeric osteopontin (Fig. 5D). These results indicate that integrin α3β1 expressed on SW480 cells is the principal integrin responsible for RGD-independent adhesion to polymeric osteopontin.

Enhanced spreading and focal contact formation of HUVE cells on polymeric osteopontin - HUVE cells were well spread on polymeric osteopontin within 30 min, while the majority of the cells on monomeric osteopontin remained round (Fig. 6A). Approximately 50% of cells had spread on polymeric osteopontin, while less than 10% of cells had spread on monomeric osteopontin (Fig. 6B). Staining of HUVE cells with fluorescein-labeled anti-phosphorylated tyrosine and phalloidin showed that HUVE cells plated on polymeric osteopontin formed focal contacts, while HUVE cells on monomeric osteopontin did not (Fig. 6C). Accumulation of phosphorylated tyrosine could be seen at the margin of the spread cells on polymeric osteopontin (Fig. 6D), which was present at the ends of actin stress fibers.

Enhanced transmigration of SW480 cells to polymeric osteopontin - Fig. 7A shows stained cells that had migrated onto the bottom of membranes coated with monomeric or polymeric osteopontin. Quantitation of these results is shown in Fig 7B. Migration on monomeric osteopontin was completely and on polymeric osteopontin was partially blocked by a combination of anti-α5β1 and αvβ5 antibodies. Transglutaminase 2 did not induce migration at the concentrarion present in the polymeric osteopontin preparation at all.

Discussion

This is the first report that directly shows biological functions of polymerized osteopontin. Previous studies have shown osteopontin is polymerized as a substrate for transglutaminase 2 *in vitro* [10,12,18] and *in vivo* [11,13,19]. However, very little is known about the functional significance of this post-translational modification. In the present study, we have shown *in vitro* with chemically polymeric recombinant osteopontin a dramatically enhanced capability for cell adhesion, migration, and spreading, which were mediated by integrins.

Polymerization might enhance interactions with integrins by concentrating ligand-binding sites, thereby enhancing integrin clustering. In fact, we observed that at a high concentration of monomeric and polymeric osteopontin (10 or 20 μg/ml), the difference of adhesion of SW480 cells to these substrates was less obvious (data not shown). However, in addition to enhancing interactions with previously described integrin receptors for osteopontin, polymerization results in new interactions with the integrins α3β1. Because osteopontin changes conformation upon polymerization [12] and is a highly flexible molecule [20,21], polymerization might expose a cryptic epitope recognized by α3β1 or induced new recognition sites.

A high molecular mass band on a polyvinylidene fluoride membrane from rat bone, immunoreactive with anti-osteopontin, was shown to contain 60% osteopontin [22]. Immunoblotting of calcified aorta from matrix Gla protein deficient mice with anti-osteopontin antibody resulted in smeared band with high molecular mass [13] like our *in vitro* polymerization. In the aorta, macrophages were present in direct contact with the polymerized osteopontin [13].

These recent works demonstrated that osteopontin is polymeric *in vivo* thus ensured that our chemically polymeric osteopontin is not specific only *in vitro*, and suggest effects of polymeric osteopontin on cellular functions.

The dramatic enhancement of biological activity of osteopontin upon polymerization mediated by integrins shed light on the mechanism by which transglutaminase 2 plays a role in cell–matrix interaction and on a new role for post-translational modification in the regulation of osteopontin function

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Figure legends

Fig. 1. Dose-dependent polymerization of recombinant osteopontin by transglutaminase 2.

A, Recombinant osteopontin was incubated with or without transglutaminase 2 in the reaction buffer. 0.5 μg of the resultant proteins were loaded into each lane of a 10% polyacrylamide gel and probed with the anti-osteopontin polyclonal antibody. Molecular mass of recombinant monomeric

osteopontin that lacks signal sequence corresponds to about 30 kDa. *B*, 0.5 μg of the purified transglutaminase 2 alone was run on a gel and probed with anti-osteopontin antibody.

Fig. 2. Adhesion of SW480 cells to monomeric and polymeric osteopontin. 5×10^4 cells in DMEM were plated on wells coated with monomeric (open square) or polymeric (closed square) osteopontin, or transglutaminase 2 (open circle) at concentrations of 0.05–1.5 μg/ml. Each plot represents the mean (± S.D.) of triplicate wells of three repeated experiments. * $p<0.05$, ** $p<0.01$ (polymeric v.s. monomeric, Student's t-test)

Fig. 3. Coating efficiency of monomeric and polymeric osteopontin. 30 μl of coating solutions of monomeric and polymeric osteopontin (10 μg/ml) before and after 24 hr incubation were subjected to western blotting with anti-osteopontin polyclonal antibody. The density was quantified by the ImageJ software and coating efficiency was calculated as percent decreased after incubation.

Fig. 4. Integrin-mediated adhesion of SW480 cells to monomeric or polymeric osteopontin. SW480 cells were plated onto wells coated with 1.0 μg/ml of monomeric (*A*) or polymeric (*B*) osteopontin in the presence or absence of integrin-blocking antibodies or RGD peptide (250 μg/ml). Each bar represents the mean (± S.D.) of triplicate wells of five repeated experiments.

Fig. 5. Inhibition of SW480 cell adhesion to polymeric osteopontin by combinations of integrin antibodies. *A*, Expression of β1-integrins on SW480 cells was analyzed by flow cytometry. Filled histogram indicates control, unstained cells. *B*, The adhesion was blocked by

either anti-α1, α2, α3, or α6 in combination with anti-α5β1 and αvβ5. *C*, The adhesion was blocked by a combination of antibodies that left only one of α1, α2, α3, or α6 functional. *D*, Inhibition effects of blockade for integrin α3β1, anti-CD151. The combinations of antibodies are shown under the figure. The adhesion was represented as percent of control. The adhesion in the presence of anti-αvβ5 and α5β1 was taken as 100%. Each bar represents the mean (± S.D.) of triplicate wells.

Fig. 6. Spreading and focal contact formation of HUVE cells on monomeric and polymeric osteopontin. HUVE cells in serum-free DMEM were plated on 96-well plates coated with 1.0 μg/ml monomeric or polymeric osteopontin at a density of $5x10^3$ cells per well. *A*, Phase-contrast microscopy of the HUVE cells. *B*, Spread cells were scored for 500 cells. Each bar represents mean (± S.D.) of 3 counted areas. For confocal microscopic observation, cells were double-stained with anti-phosphotyrosine followed by Alexa Fluor 555 goat anti-mouse IgG (red), and Alexa Fluor 488 phalloidin (green) for actin filaments. *C*, Stained cells on monomeric or polymeric osteopontin. *B*, Magnified view of cells on polymeric osteopontin showing the location of phosphorylated tyrosine and actin filaments. 20-μm scale bars are indicated within the panels.

Fig. 7. Increased transmigration of SW480 cells to polymeric osteopontin and its inhibition by anti-integrin antibodies. *A*, Microscopic view of stained cells transmigrated onto the bottom of the membrane without any blockade. *B*, Effects of integrin-blocking antibodies, anti-α5β1, and -αvβ5. Cells migrated in the presence or absence of the blocking antibodies (open column, antibody (-); gray column, anti-α5β1 and αvβ5) were counted for 3 microscopic areas. Each bar represents mean (± S.D.) of the areas of the 3 repeated experiments.

Fig.1

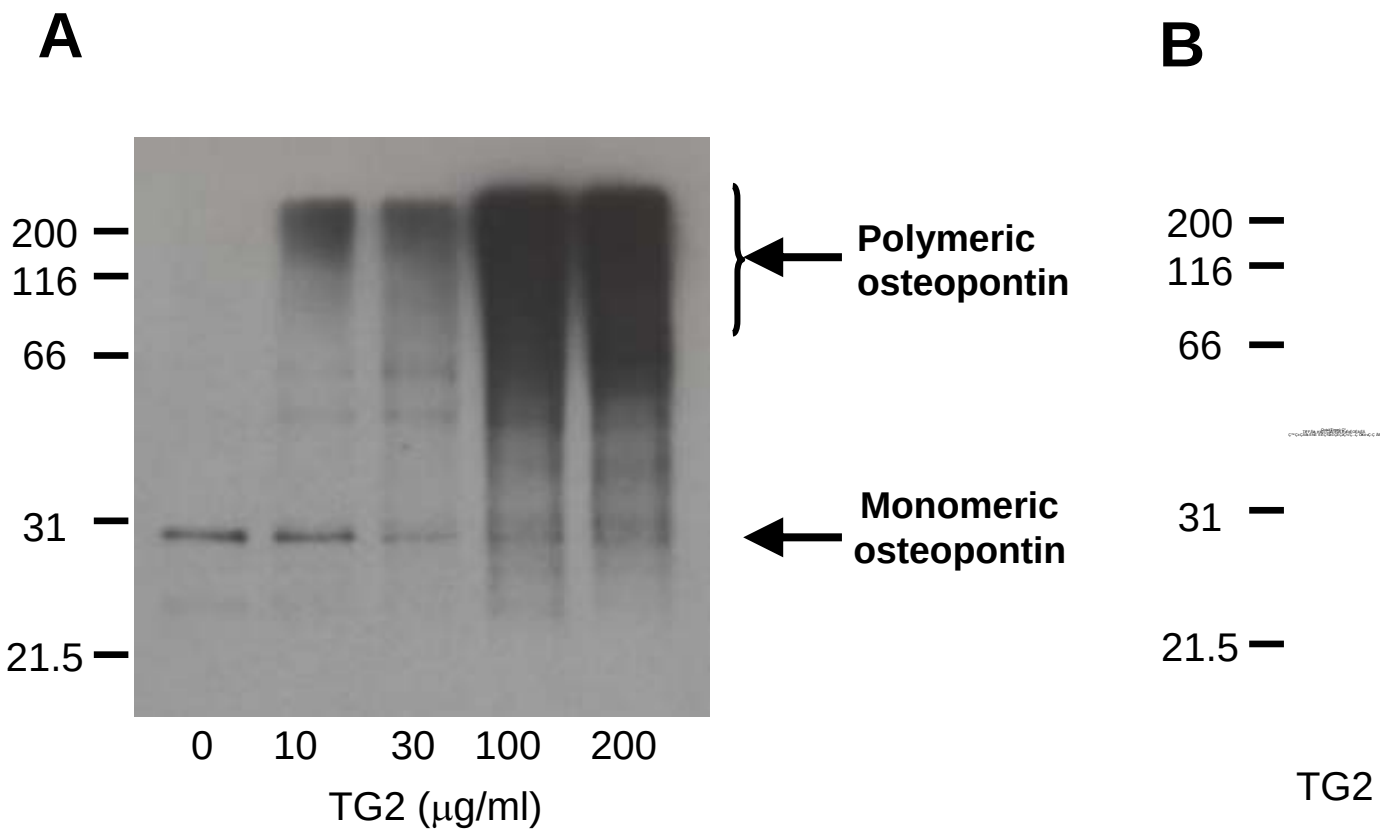
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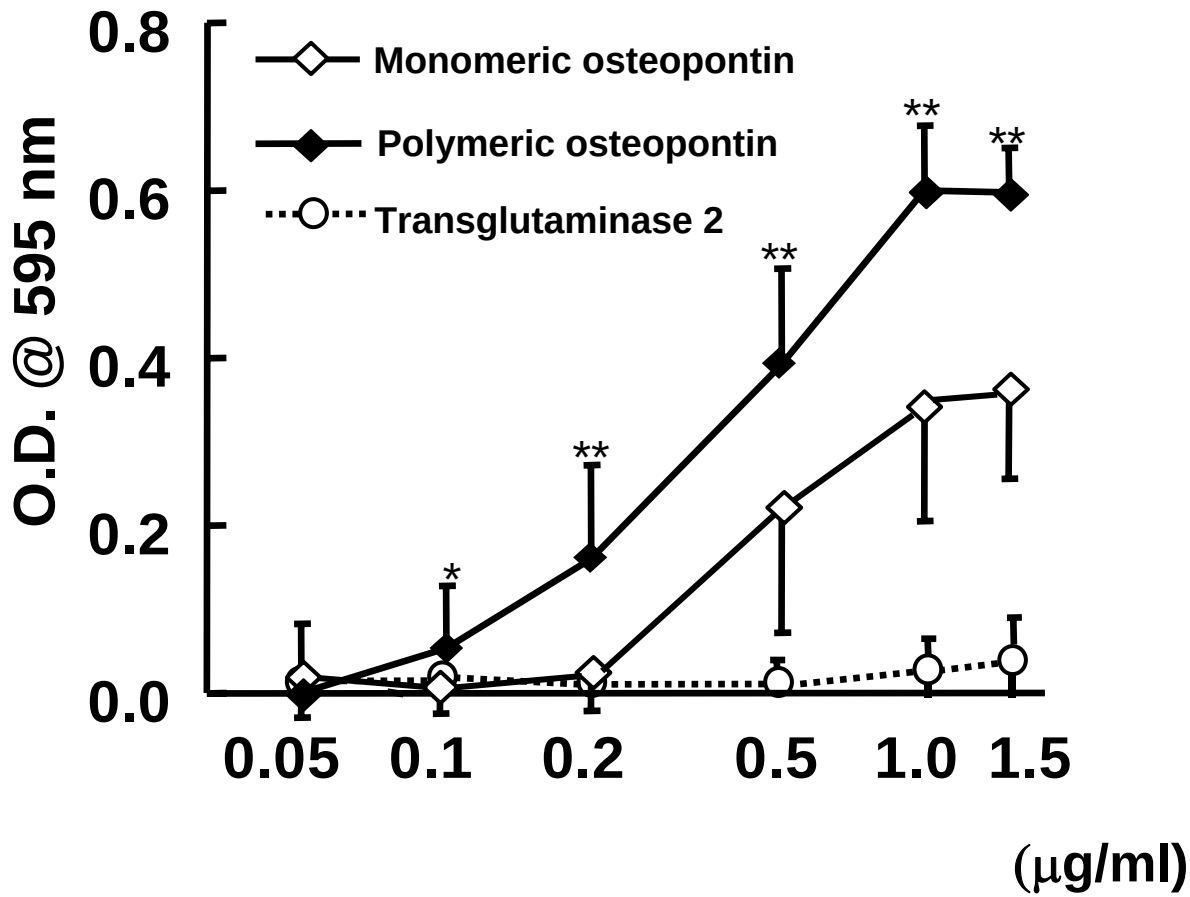
Fig.2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Polymeric osteopontin
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Fig.3

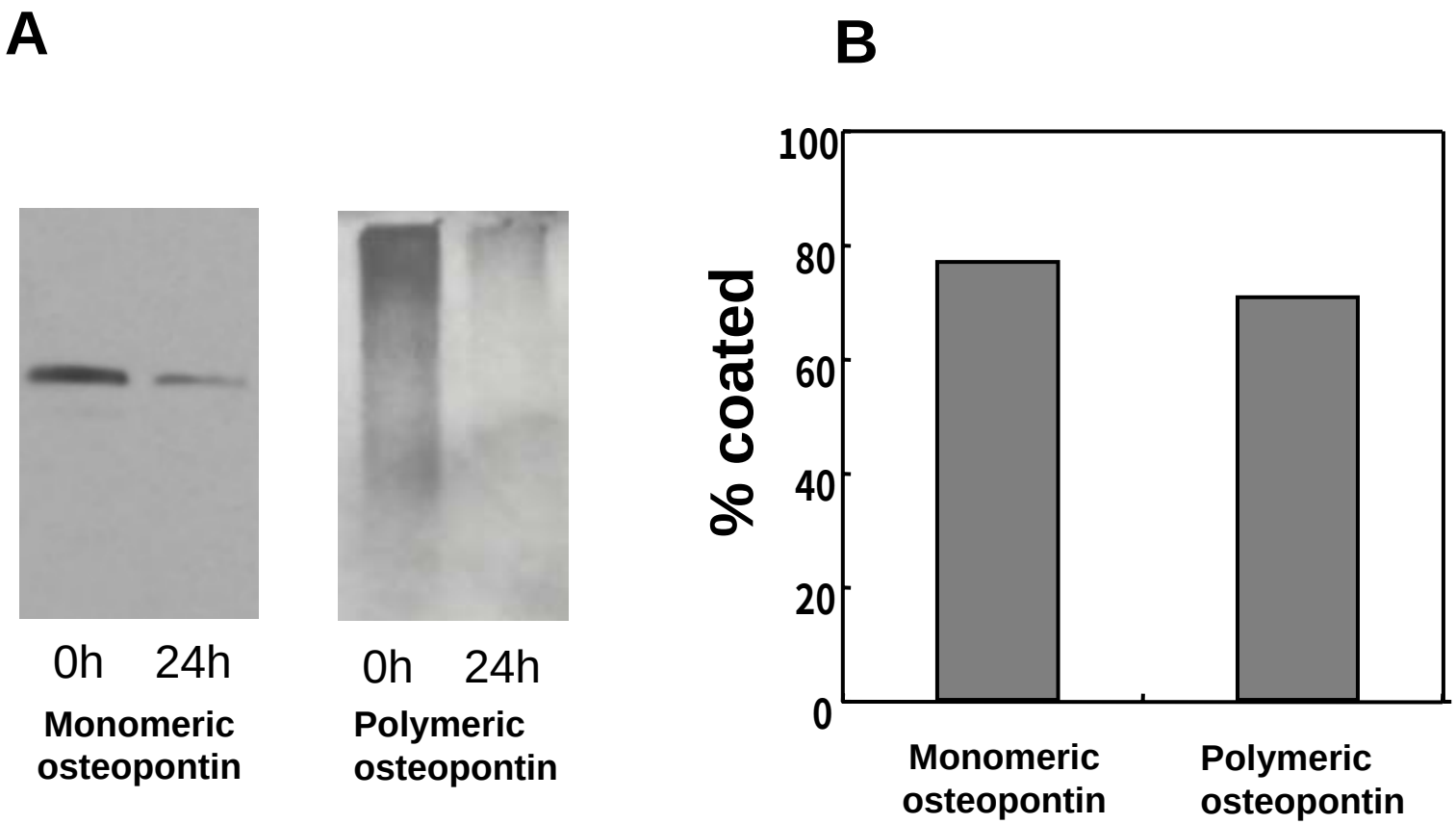
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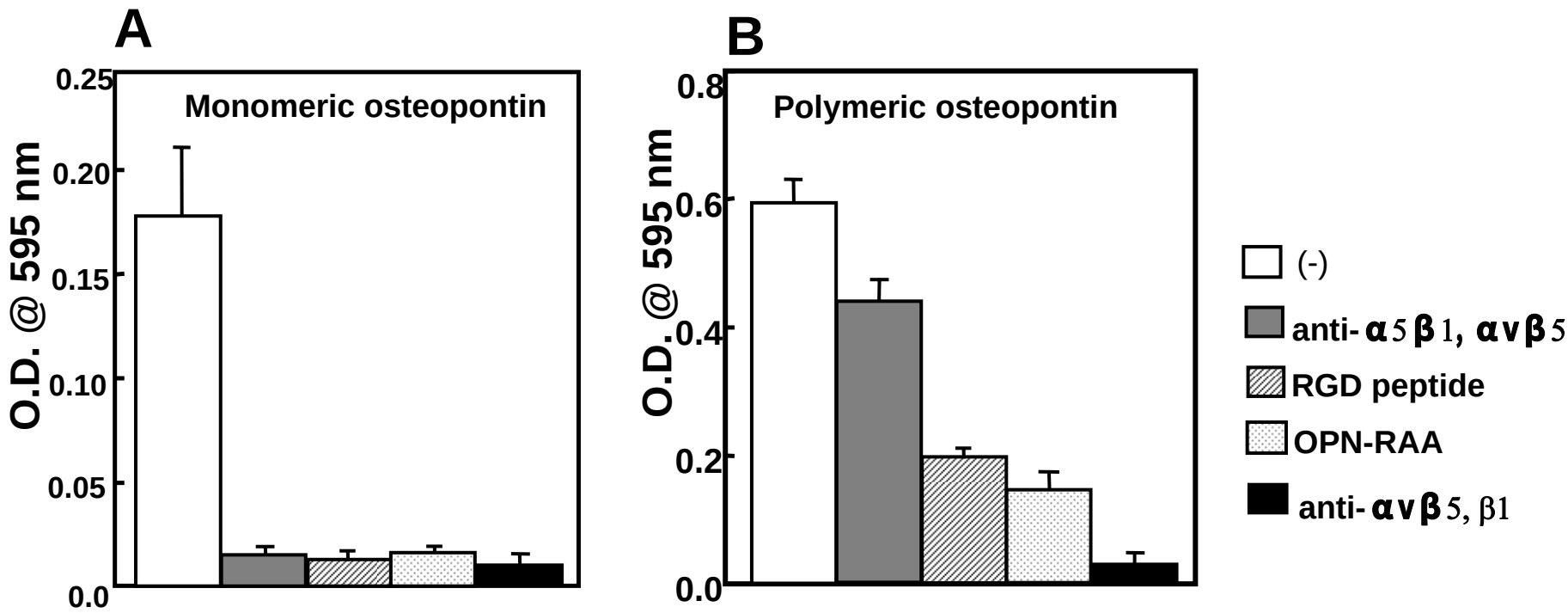
Fig.4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OPN-RAA
anti-αvβ5, β1

Fig.5

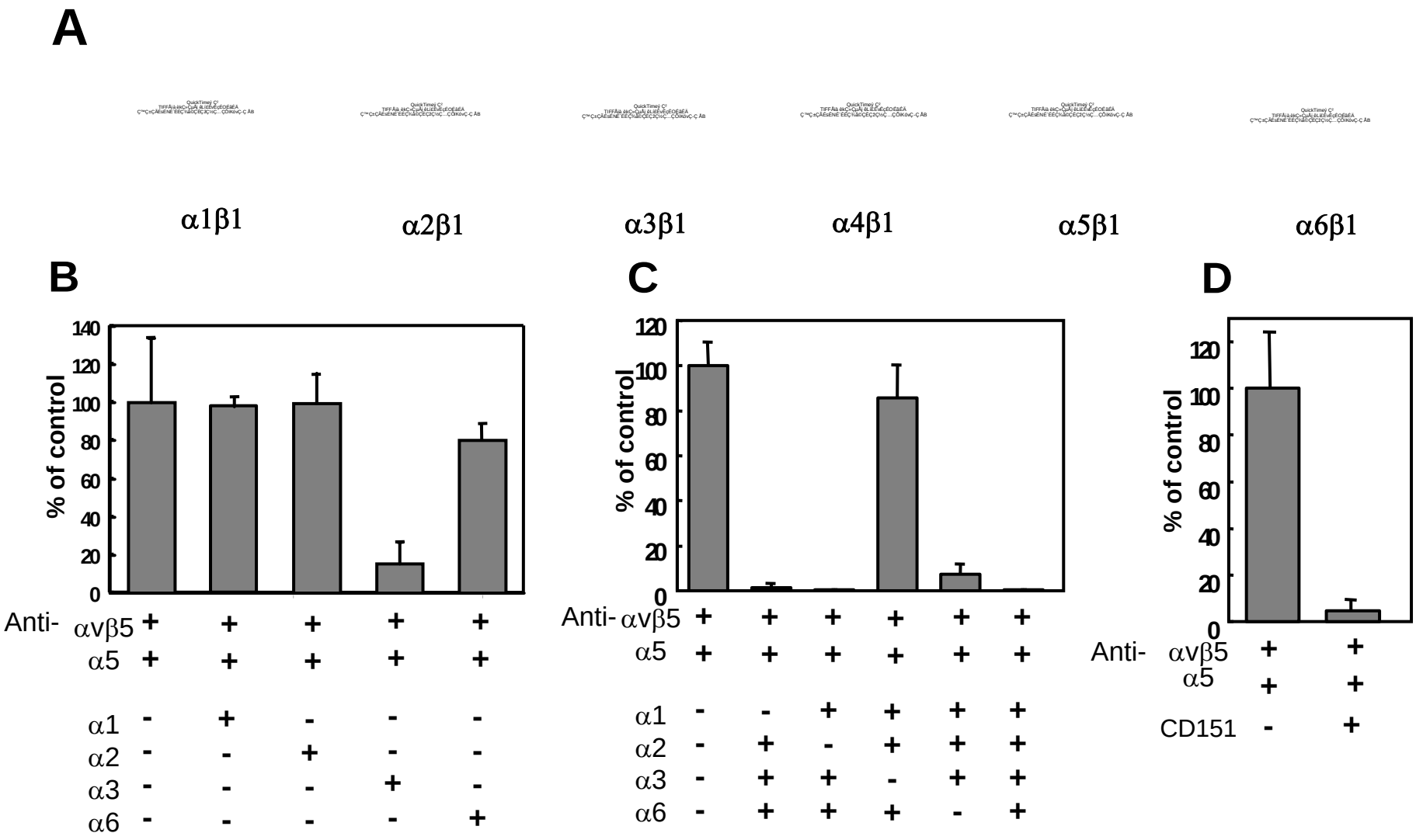
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Fig.6

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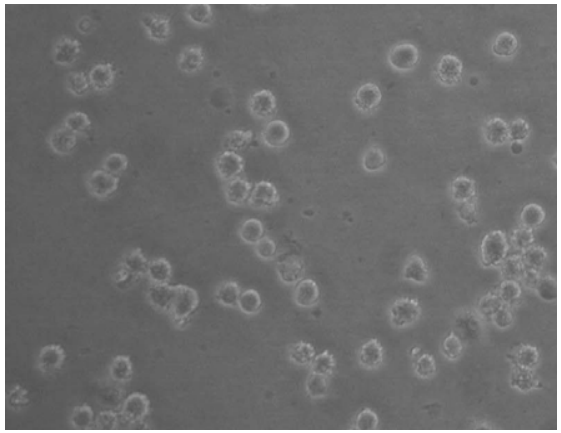
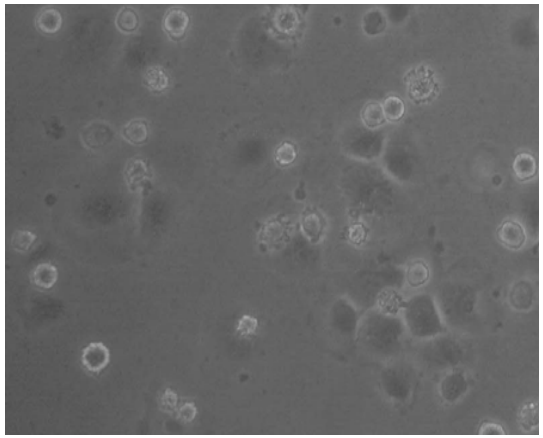
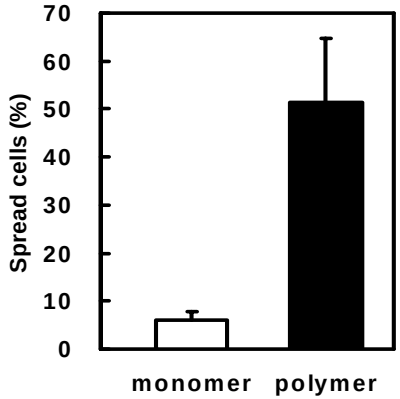


Monomeric osteopontin

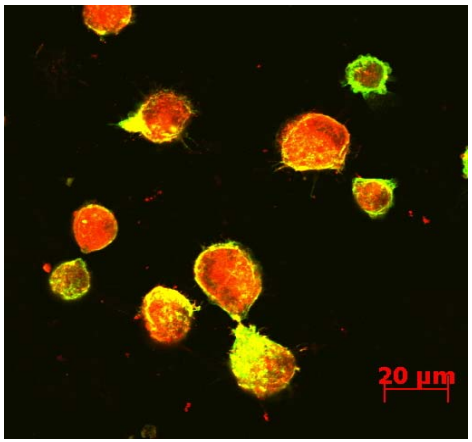


Polymeric osteopontin

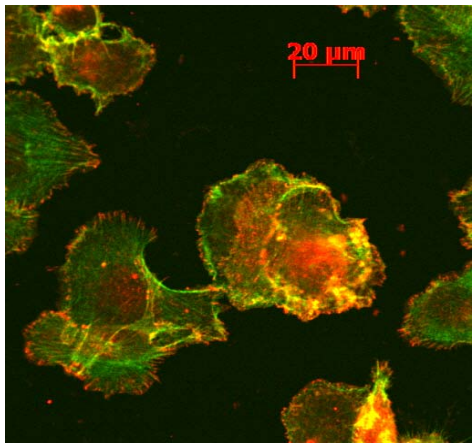
B



C

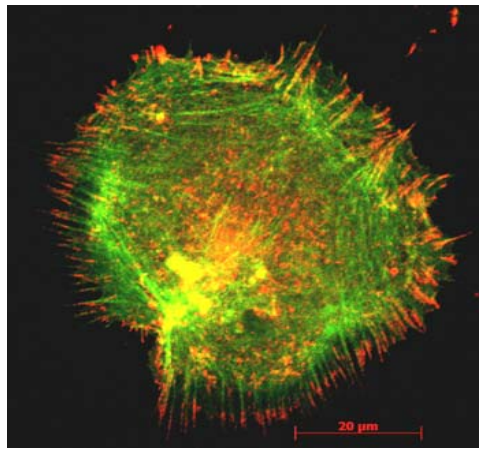


Monomeric osteopontin



Polymeric osteopontin

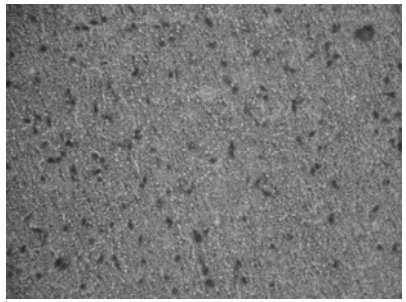
D



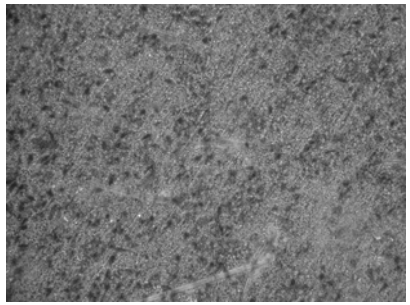
Polymeric osteopontin

Fig.7

A



Monomeric osteopontin



Polymeric osteopontin

B