Synergistic Growth Stimulation of Mouse Fibroblasts by Tumor-derived Adhesion Factor with Insulin-like Growth Factors and Insulin

Kotaro Akaogi, Junji Sato, Yukie Okabe, Yoshimasa Sakamoto, Hidetaro Yasumitsu, and Kaoru Miyazaki

Division of Cell Biology, Kihara Institute for Biological Research, Yokohama City University, 641-12 Maoka-cho, Tosuka-ku, Yokohama 244, Japan

Abstract
Tumor-derived adhesion factor (TAF) has been purified as a major secretory protein with cell-adhesion activity from culture medium conditioned by human bladder carcinoma cells (K. Akaogi et al., Biochem. Biophys. Res. Commun., 198: 1046-1053, 1994). TAF is closely related or identical to a putative protein encoded by the mac25 gene (M. Murphy et al., Cell Growth & Differ., 4: 715-722, 1993) and prostacyclin-stimulating factor (T. Yamauchi et al., Biochem. J., 303: 591-596, 1994). TAF has a characteristic cysteine cluster in its NH2-terminal sequence, which is conserved in the NH2 terminus of insulin-like growth factor-binding proteins. In the present study, we examined the interaction of TAF with insulin-like growth factors (IGFs) and insulin. Although in a ligand blotting assay TAF bound neither IGFs nor insulin, immunoprecipitation assay showed weak interaction of TAF with IGF-I, IGF-II, and insulin. TAF by itself stimulated the growth of BALB/c3T3 cells on the usual plastic substrates and significantly enhanced IGF- or insulin-mediated cell growth. Moreover, marked potentiation of the mitogenic activities of IGFs and insulin by TAF was found in the culture on type IV collagen substrates. The synergistic growth stimulation was completely blocked by heparin. These results suggest that TAF may regulate the growth of fibroblastic cells in collaboration with IGFs and insulin.

Introduction
IGFs and insulin are structurally related peptides that mediate a large number of similar cellular processes, such as activation of nutrient transport, modulation of metabolic enzyme activities, and stimulation of cell proliferation (1, 2). IGFs circulate in plasma and other body fluids as high molecular weight complexes with specific binding proteins, IGFBPs (3). Six distinct IGFBPs have been identified in a wide variety of biological fluids and conditioned media of cultured cells (4-7). IGFBPs regulate IGF availability in tissues and body fluids and modulate the mitogenic activity of IGFs. Plasma IGFBPs increase the half-life of IGFs in circulation. Isolated IGFBPs have been shown to inhibit or potentiate IGF actions in various experimental systems in vitro (8-12). Every mature protein of IGFBPs has 18 highly conserved cysteine residues, 12 of which are located in the NH2-terminal region and thought to be involved in IGF binding (13).

Recently, we purified a major secretory protein with a molecular weight of about M, 30,000 from the culture of human bladder carcinoma cell line EJ-1 (14). Because this protein had cell-adhesion activity, it was tentatively named TAF. The structural analysis indicated that TAF was closely related or identical to a putative protein encoded by the mac25 gene, the cDNA of which had been cloned from leptomenigeal cells (15). The expression of the mac25 gene has been reported to be down-regulated in meningiomas compared to the normal counterpart (15) and up-regulated in senescent human mammary epithelial cells (16). The NH2-terminal sequence of the predicted mac25 protein contains a cluster of 11 cysteine residues, which is conserved among IGFBPs. The predicted amino acid sequence of the mac25 protein has 23% identity in average and 40-45% similarity to IGFBPs (16). Although the amino acid identity between mac25 protein and IGFBPs is far lower than that among IGFBPs (<40%), the highly conserved cysteine cluster in the NH2 terminus of mac25 suggests the possibility that it may act as an IGFBP. On the other hand, Yamauchi et al. (17) recently identified a protein that stimulated prostacyclin production in vascular endothelial cells from conditioned medium of human fibroblasts and cloned its cDNA. The deduced sequence of the PSF is essentially identical to that of mac25, with the exception of several amino acid residues in its NH2-terminal and COOH-terminal amino acid sequences. Our cDNA cloning and amino acid sequence analysis of TAF have shown that the deduced sequence of TAF is almost identical to that of PSF. Very recently, we have found that TAF has type IV collagen-binding activity and is densely
accumulated in the vascular basement membrane of human tumor tissues and in capillary tube-like structures of cultured vascular endothelial cells (18). This suggests the possible involvement of TAF in tumor angiogenesis.

In the present study, we examined the possible function of TAF as an IGFBP. TAF appeared to have low affinity to IGF-I, IGF-II, and insulin and exerted a synergistic growth-stimulatory effect with IGFs and insulin on BALB/c3T3 fibroblasts.

Results
Interaction of TAF with 125I-labeled IGF-I, IGF-II, or Insulin. IGFBPs consist of three distinct domains each comprising approximately one-third of the molecule. Most human IGFBPs contain 12 and 6 invariant cysteine residues in the NH2-terminal (domain 1) and the COOH-terminal (domain 3) regions, respectively. Both cysteine clusters are thought to be involved in IGFBinding. TAF/mac25/PSF also contains 18 cysteine residues in the molecule, of which 11 residues in the NH2-terminal region are well conserved among IGFBPs (Fig. 1).

IGFBPs are known to strongly bind IGF-I and IGF-II in the ligand blotting analysis. We assayed the interaction of TAF with IGF-I, IGF-II, and insulin by the ligand blotting analysis, in which the 125I-labeled factors were incubated with TAF immobilized on the nitrocellulose membrane. In this assay, however, no specific band was detected. Next we attempted to detect relatively weak interaction by immunoprecipitation. TAF was incubated with 125I-labeled IGF-I, IGF-II, or insulin, and the complex formed was cross-linked with DSS, followed by immunoprecipitation with an anti-TAF monoclonal antibody. The immunoprecipitate was analyzed by SDS-PAGE and subsequent autoradiography. As shown in Fig. 2, Lane 1, the incubation of TAF with 125I-labeled IGF-I or 125I-labeled insulin formed the complex at an approximate molecular weight of M, 37,000. In addition, free forms of 125I-labeled IGF-I (M, 7600) and 125I-labeled insulin (M, 6000), which were probably derived from non-cross-linked complexes, were detected on the gel. These high and low molecular weight bands were not detected in the incubation without TAF (data not shown). Furthermore, the complex of TAF and 125I-labeled IGF-I was undetectable when the reaction mixture was added with an excess amount of unlabeled IGF-I (Fig. 2, left Lane 2) or insulin (Fig. 2, left Lane 3) but detected with soybean trypsin inhibitor as a negative control (Fig. 2, left Lane 4). Similarly, the cross-linking of TAF with 125I-labeled insulin was prevented by an excess amount of unlabeled insulin (Fig. 2, right Lane 2) or IGF-I (Fig. 2, right Lane 3) but not by soybean trypsin inhibitor (Fig. 2, right Lane 4). When TAF was incubated with 125I-labeled IGF-I, essentially the same results were obtained as in the case of 125I-labeled IGF-I (data not shown). These results indicate that the binding of TAF with IGF-I, IGF-II, or insulin is specific, and that TAF has a common binding site for these peptide factors.

It has been reported that insulin binds to type I IGF receptor but not to IGFBPs (19). Therefore, the binding of TAF to

Fig. 1. Amino acid homology between NH2-terminal sequences of the mature form of TAF and IGFBP-1. Boxed residues indicate highly conserved cysteine residues found in IGFBPs. The NH2 terminus of TAF corresponds to residue 27 of the proforms of TAF/mac25/PSF. The NH2 terminal of mac25 protein and PSF have not been determined.

Lane 3) but detected with soybean trypsin inhibitor as a negative control (Fig. 2, left Lane 4). Similarly, the cross-linking of TAF with 125I-labeled insulin was prevented by an excess amount of unlabeled insulin (Fig. 2, right Lane 2) or IGF-I (Fig. 2, right Lane 3) but not by soybean trypsin inhibitor (Fig. 2, right Lane 4). When TAF was incubated with 125I-labeled IGF-I, essentially the same results were obtained as in the case of 125I-labeled IGF-I (data not shown). These results indicate that the binding of TAF with IGF-I, IGF-II, or insulin is specific, and that TAF has a common binding site for these peptide factors.

It has been reported that insulin binds to type I IGF receptor but not to IGFBPs (19). Therefore, the binding of TAF to
insulin immobilized on a plastic plate was also examined by ELISA with anti-TAF antibody. As shown in Fig. 3, TAF bound to immobilized insulin in a concentration-dependent manner but not to BSA. The affinity constant ($K_d$) of TAF for insulin was calculated to be $3.7 \times 10^{-8}$ M by the Scatchard analysis. These three different assays suggest that TAF has some affinity to both IGFs and insulin, although it is far lower than those of IGFBPs for IGFs. Most of IGFBPs are known to have $K_d$ values for IGF-I and IGF-II between 3.0 and $5.9 \times 10^{-11}$ M (20).

**Effects of TAF on IGF-I- and Insulin-stimulated Cell Growth.** To understand the biological significance of the interaction between TAF and IGFs or insulin, their effects on cell growth were investigated. The BALB/c3T3 mouse fibroblast cell line was chosen for this study because of its well-characterized response to IGFs and insulin (21, 22). BALB/c3T3 cells hardly grew in a culture medium containing 0.5% FCS on the usual plastic substrates. IGF-I and insulin stimulated the growth of BALB/c3T3 cells under these conditions, resulting in 5- and 3-fold increases of cell number compared to the control, respectively (Fig. 4A). Interestingly, TAF at 1 $\mu$g/ml stimulated the cell growth to a slightly higher extent than insulin (Fig. 4A). When TAF, in addition to IGF-I or insulin, was added to the culture, additional growth stimulation was obtained. TGF-β, which did not interact with TAF, weakly stimulated cell growth, but the additional growth-stimulatory effect of TAF for this growth factor was lower compared to the cases of IGF-I and insulin (Fig. 4A). EGF showed a growth effect similar to that of TGF-β in the absence and presence of TAF (Fig. 4A).

We have found that TAF specifically binds to type IV collagen, and that it promotes adhesion of vascular endothelial cells to type IV collagen (18). Therefore, we also tested the growth-stimulatory effect of TAF on type IV collagen substrates in the absence and presence of IGF-I or insulin (Fig. 4B). When BALB/c3T3 cells were plated on type IV collagen-coated plates, growth stimulation by IGF-I, insulin, or TAF alone was significantly lower than that on non-coated plastic plates. However, when both TAF and IGF-I were added to the culture, the cell growth was enhanced about 14-fold compared with the control (Fig. 4B). The growth effects of the two factors seemed synergistic, because either IGF-I or TAF alone induced the cell growth only 3.1- and 3.4-fold, respectively. A similar result was observed when both TAF and insulin were added to the culture (Fig. 4B). TAF also showed a synergistic growth effect with IGF-II on type IV collagen substrates but to a slightly lower extent than that with insulin (data not shown). To examine whether the synergism of TAF was limited to IGFs and insulin, its synergistic effect with TGF-β and EGF was examined. Although TAF further enhanced the TGF-β- or EGF-mediated cell growth, the synergistic enhancement of cell growth was not evident (Fig. 4B).

To show the synergistic effect of TAF with IGF-I more clearly, checkerboard analysis for growth of BALB/c3T3 cells was carried out with different concentrations of TAF and IGF-I on type IV collagen substrates (Fig. 5). In the absence of the other factor, TAF and IGF-I individually stimulated the cell growth in a concentration-dependent manner, and the growth stimulation was significantly higher with IGF-I than with TAF. When both factors were simultaneously added to
the culture, the growth stimulation was much higher than that expected from the simple addition of their growth stimulatory effects at any point. For example, 0.2 μg/ml TAF and 1 ng/ml IGF-I increased the cell number by 20 and by 46%, respectively, in their separate addition, whereas their simultaneous addition increased the cell number by 254%. These results indicate that TAF potentiates the mitogenic activity of IGF-I on type IV collagen substrates. The growth stimulation by IGF-I was almost saturated at a concentration of 50 ng/ml, and even at 250 ng/ml essentially the same levels of cell growth as those at a concentration of 50 ng/ml were obtained in both the absence and presence of TAF (data not shown). This suggests that TAF may not affect the availability of IGF-I to the cell surface receptor.

We have reported that heparin inhibits the binding of TAF to a cell surface receptor (18). To get a clue for the mechanism by which TAF potentiates the mitogenic activity of IGF-I, the effect of heparin on cell growth was examined in the presence of IGF-I and/or TAF on the type IV collagen substrates (Fig. 6). Heparin slightly suppressed the growth of BALB/c3T3 cells in the absence and presence of either IGF-I or TAF. More importantly, heparin completely inhibited the synergistic growth stimulation by IGF-I and TAF. This indicates that the direct binding of TAF to cells is required for the synergistic growth stimulation by IGF-I and TAF.

Morphology of BALB/c3T3 cells cultured in the presence of insulin and/or TAF on type IV collagen substrates is shown in Fig. 7. In the absence of TAF (Fig. 7, A and C), some cells showed rather elongated morphology, whereas in the presence of TAF (Fig. 7, B and D), most cells showed more spread morphology. It has been shown previously that TAF promotes adhesion of various kinds of cells to plastic substrates in serum-free medium (14). In the presence of 0.5% FCS, however, BALB/c3T3 cells adhered well to the non-coated or type IV collagen-coated substrates, regardless of the presence or absence of TAF. Therefore, it appeared unlikely that the growth-stimulatory effect of TAF shown in Fig. 4 totally depended on its cell adhesion activity.

Discussion

Six kinds of human IGFBPs have high sequence homology to each other in their NH₂-terminal and COOH-terminal regions, both of which have a consensus cysteine cluster. Some studies showed the contribution of the NH₂-terminal region of IGFBPs for IGF binding (13), whereas others claimed the

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### Table 1

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<tr>
<th>TAF (μg/ml)</th>
<th>0</th>
<th>0.2</th>
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<td>50</td>
<td>422</td>
<td>1051</td>
<td>1110</td>
<td>1567</td>
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Relative cell number (%)
importance of the COOH-terminal region for the binding (23). TAF has a similar NH2-terminal cysteine cluster to that in IGFBPs, but there is little sequence homology in the central and COOH-terminal regions between TAF and IGFBPs. IGFBPs specifically and strongly bind IGF-I and IGF-II. Insulin binds to the type I IGF receptor but not to IGFBPs (19), and its biological activity is not affected by IGFBPs (9, 12). In the present study, affinity cross-linking assay and microparticle plate assay showed that TAF weakly bound not only IGF-I and IGF-II but also insulin. In the ligand blotting assay, however, TAF could not bind the growth factors. These results suggest that the NH2-terminal cysteine cluster in TAF and IGFBPs contributes to the recognition of IGF-I, IGF-II, and insulin, whereas the COOH-terminal common structure of IGFBPs is responsible for the high affinity binding to the two IGFs and for the discrimination between IGFs and insulin. The affinity of TAF for insulin ($K_d = 3.7 \times 10^{-8} \text{M}$) is about 1000 times lower than those of IGFBPs for IGF-I and IGF-II ($K_d = 3.0-5.9 \times 10^{-11} \text{M}$). These results demonstrate that TAF is a distinct protein from the IGFBP family. At this point, it is not clear whether the weak interaction of TAF with IGF-I, IGF-II, and insulin has biological significance.

IGFBPs are known to inhibit or enhance the mitogenic activity of IGFs, depending on the experimental conditions (8–12). Soluble IGFBPs inhibit the activity of IGFs by preventing the IGF binding to the cell surface receptor (10, 24). On the other hand, when IGFBPs are bound to the cell surface, they enhance the mitogenic activity of IGFs by increasing the IGF binding to the receptor (10, 11). In the present study, we also found that TAF by itself stimulated the growth of BALB/c3T3 cells in low serum medium. When IGF-I or insulin in addition to TAF was added to the culture on plastic substrates, an additive growth-simulating effect of the two factors was obtained. More importantly, when the cells were plated on type IV collagen-coated plates, TAF potently enhanced the IGF-I or insulin-stimulated cell growth. This synergistic or cooperative growth stimulation by TAF seemed specific for IGFs or insulin, because such effect was not evident with the combination of TAF with either TGF-β or EGF. Although the exact mechanism for the synergism of TAF and IGFs/insulin is unknown, several possibilities can be considered.

(a) The weak interaction of TAF with IGFs/insulin and their apparently specific synergism for cell growth suggest the possible function of TAF as a reservoir for IGFs and insulin. It has been reported that IGFBP-5 binds to fibroblast-produced ECM, including type III and IV collagens, laminin and fibronectin, and potentiates the effect of IGF-I on fibroblast growth (25). Similarly, TAF bound to the type IV collagen substrate or cell surface may function as a reservoir for IGFs and insulin. IGFBP-3 is proteolytically processed to a low molecular weight form to have a 10 times lower affinity for IGF-I compared to the soluble form (26). ECM-associated IGFBP-5 also has a 7-fold decreased affinity for IGF-I compared to IGFBP-5 in solution (25). It is conceivable that the lowered affinity of the IGFBPs for IGF-I makes it easier to transfer the IGF molecules from the cell- or ECM-associated IGFBP to the IGF receptor. The low affinity of TAF for these growth factors may be favorable to effective transfer of TAF-bound IGFs or insulin to the cell surface receptors. However, this possibility does not seem so high, because the experiment with heparin indicated that the direct binding of TAF to cells was required for the synergistic growth stimulation, and because the synergistic growth stimulation of TAF was observed even when an excess amount of IGF-I was added to the culture.

(b) The cell adhesion activity of TAF may contribute to the synergistic growth stimulation. TAF at concentrations higher than 5 μg/ml stimulated the adhesion of BALB/c3T3 cells to plastic plates in serum-free medium (data not shown), but these cells attached well to and spread on the plastic substrates in the TAF-free and 0.5% FCS-containing medium, which was used as a basal medium for growth experiments. In addition, the growth stimulation by TAF was observed at concentrations below 1 μg/ml, where its cell adhesion activity was undetectable. These facts make the second possibility unlikely.

(c) TAF may directly stimulate cell growth, and its signal pathway may synergistically cross-talk with that induced by IGFs or insulin but not by EGF and TGF-β. The growth stimulation by TAF itself seemed not to be mediated by IGF-I or insulin, because it was blocked neither with anti-IGF-I antibody nor with anti-insulin antibody in the absence of exogenous IGF-I or insulin (data not shown). TAF is probably identical to PSF, which was identified as a protein that stimulated prostacyclin (PGI2) release from vascular endothelial cells (17). Prostacyclin is induced by various growth factors such as EGF and platelet-derived growth factor. Therefore, it is likely that TAF acts as a growth factor. However, it still remains unknown why type IV collagen enhances the growth-stimulating effect of TAF and IGFs/insulin. Type IV collagen may support the binding of TAF to cells as a reservoir for TAF. It also seems possible that type IV collagen stimulates the production of IGF receptors or a TAF receptor in BALB/c3T3 cells. These possibilities should be further tested to clarify the mechanism for the unique growth-stimulatory activity of TAF.

TAF is produced by normal endothelial cells and fibroblasts as well as various human cancer cell lines in vitro.6 TAF is densely accumulated in vascular basement membrane, probably binding to type IV collagen, in tumor tissues but hardly in normal tissues (18). Previously, we failed to show growth-stimulating activity of TAF toward bovine capillary endothelial cells (14). In that assay, TAF was added to the culture with 10% FCS-containing medium, because of their high serum dependency for cell growth and survival. The apparent insensitivity of the endothelial cells to TAF seems due to the presence of serum, because even BALB/c3T3 cells did not respond to TAF in the 10% FCS-containing medium (data not shown). It seems very likely that the growth-stimulating mechanism of TAF described above contributes to the local growth of fibroblasts, endothelial cells, and other types of cells under various pathological conditions, such as wound healing and tumor angiogenesis.

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Materials and Methods

Purification of TAF. TAF was purified to homogeneity by Reactive Red-agarose affinity chromatography, anion-exchange chromatography, cat-ion-exchange chromatography, and C4 reverse-phase high-performance liquid chromatography from the serum-free conditioned medium of EJ-1 cells, according to the procedure described before (14). Purified TAF stimulated the adhesion of BALB/c 3T3 cells to plastic substrates at a concentration of 2.5 μg/ml in serum-free culture medium.

Electrophoretic Analysis. SDS-PAGE was carried out on 14% gels under nonreducing conditions by the method of Laemmli (27). Proteins on the gels were stained with Coomassie Brilliant Blue R-250 or with a Wako silver staining kit (Wako Chemical, Osaka, Japan).

Iodination of IGFs and Insulin. IGF-I, IGF-II, and insulin were iodinated with 125I by the chloramine-T method (28). Briefly, 10 μg of each peptide were iodinated with 500 μCi of Na125I. Iodinated peptides were separated from free Na125I by gel filtration through a 2-6DG column (Bio-Rad, Richmond, CA). Specific activities of 125I-labeled IGF-I, IGF-II, and insulin were 12.2, 10.6, and 5.36 μCi/μg, respectively.

Affinity Cross-Linking and Immunoprecipitation. Two hundred ng of TAF was incubated overnight at room temperature with 40 ng of 125I-labeled IGF-I, IGF-II, or insulin in 50 μl of Dulbecco’s Ca2+- and Mg2+-free PBS. The resultant complex of 125I-labeled IGF-I, 125I-labeled IGF-II, or 125I-labeled insulin with TAF was incubated with 0.275 μw DSS as a cross-linking reagent at room temperature for 20 min, followed by termination with 50 μw Tris-HCl (pH 7.5) and 100 μw glycine. For immunoprecipitation, 1.25 μl of anti-TAF monoclonal antibody (no. 88) was added to the reaction mixture and incubated overnight at room temperature. The antibody-bound complex formed was precipitated with protein A-Sepharose beads pretreated with rabbit antiserum IgG antisera. The resultant precipitates were washed five times with 200 μw Tris-HCl (pH 7.5), 0.15 μw NaCl, and 1% Triton X-100, incubated in Laemmli’s SDS sample buffer without a reducing reagent at 100°C for 5 min, and then subjected to SDS-PAGE. Gels were dried and examined by autoradiography using Kodak X-OMAT film.

Binding Assay of TAF to Insulin Immobilized on Plastic Plates. Each well of ELISA plates (Costar, Cambridge, MA) was incubated with 50 μl of insulin (40 μg/ml) in 0.1 ml sodium carbonate hydroxide (pH 9.6) at 37°C for 1.5 h and blocked with 1% BSA in PBS at 37°C for 1 h. The insulin-coated wells were added with various concentrations of TAF in 50 μl of PBS and incubated overnight at 4°C. Then, the primary antibody against TAF (dilution, 1:10000) was added to the wells and incubated at 37°C for 1.5 h. The antibody-treated wells were sequentially incubated at room temperature for 45 min with biotinylated rabbit antiserum IgG (Vector, Burlington, CA) and with avidin-alkaline phosphatase (Vector; dilution, 1:200). The color was developed by 1 mg/ml p-nitrophenylphosphate in 100 μw diethanolamine (pH 9.6) containing 0.24 μw MgCl2 and measured for absorbance at 405 nm with Immunoreader NJ-2000 (Inter Med, Tokyo, Japan).

Cell Line and Culture Conditions. BALB/c3T3 fibroblasts were maintained in a DMEM/F12 medium (Life Technologies, Inc., Grand Island, NY) supplemented with streptomycin sulfate, penicillin G, and 10% FCS (Mongagua, Melbourne, Australia; DMEM/F12 + 10% FCS) at 37°C in 5% CO2. Plastic culture dishes were purchased from Sumible Medical (Tokyo, Japan).

Cell Proliferation Assay. Each well of 24-well plastic culture plates was incubated with 100 μg/ml of type IV collagen in PBS at 37°C for 1.5 h and washed twice with PBS. BALB/c3T3 cells were inoculated at a density of 5 × 103 cells in 0.5 ml of DMEM/F12 + 0.5% FCS on the type IV collagen-coated plates or on the non-coated plates. Test samples were added at the time of inoculation of the cells. After incubation for 3 days, the number of cells was determined with an automatic cell counter (Coulter Electronics, Beds, England).

Reagents. Human recombinant IGF-I and human recombinant TGF-β1 were purchased from Austral Biologicales (San Ramon, CA); human IGF-I was from Upstate Biotechnology, Inc. (Lake Placid, NY); BSA was from Sigma Chemical Co. (St. Louis, MO); and bovine pepsin-treated lens type IV collagen was from Iwaki Glass (Tokyo, Japan). Anti-TAF monoclonal antibody (no. 88) was prepared in our laboratory.

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References


