Effect of tenascin-X together with vascular endothelial growth factor A on cell proliferation in cultured embryonic hearts.

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Tenascin-X (TNX) is a large glycoprotein that appears in extracellular matrices. Previously, we demonstrated that TNX binds to vascular endothelial growth factors A and B (VEGF-A and -B) and that VEGF-B in combination with TNX induces DNA synthesis in endothelial cells via increased signals mediated by the VEGFR-1 receptor. In this study, we investigated the effect of TNX with VEGF-A on the cell proliferation in embryonic mouse heart explants from either wild-type (TNX+/+) or TNX-deficient (TNX−/−) mice. The addition of VEGF-A to the explants from TNX+/+ mice increased cell proliferation by 1.5 fold compared with that in TNX−/− mice, indicating that TNX with VEGF family member plays an important role in the control of endothelial cell proliferation in vivo.

Key words  tenasin-X; vascular endothelial growth factor A; extracellular matrix

MATERIALS AND METHODS

Materials  Biochemical reagents were from Wako Pure Chemical Industries (Osaka, Japan). Oligonucleotides were from Hokkaido System Science (Sapporo, Japan). Collagen gel was from Nitta Gelatin Co., Ltd. (Osaka). Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F12 medium were from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Eagle’s minimal essential medium (MEM) was from Gibco BRL (Tokyo). Fetal bovine serum (FBS) was from Dainippon Pharmaceutical Co., Ltd. (Osaka). Oligonucleotides were from Amersham Pharmacia Biotech (Tokyo). Human VEGF-A165 was from Gibco BRL (Tokyo). The expressed sequence tag (EST) clone (Image clone ID, 1690025; GenBank accession no. AI122772) encoding human basic fibroblast growth factor (bFGF) was purchased from Incyte Genomics, Inc. (St. Louis, U.S.A.).

Animals  The generation of tenasin-X-deficient (TNX−/−) mice by homologous recombination using embryonic stem (ES) cells has been described. The genetic background of TNX−/− mice is a mixture of C57BL/6, CBA and ICR strains. The control wild-type ICR (TNX+/+) mice were purchased from Sankyo Labo Service Co., Ltd. (Tokyo, Japan). The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used at 8 weeks of age.

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Preparation of Recombinant Mouse VEGF-B186 and Human bFGF  Mouse VEGF-B186 cDNA clone was described in a previous paper. Human bFGF cDNA was purchased from Incyte Genomics, Inc. (St. Louis, U.S.A.). Each cDNA was subcloned into the GEX-6P vector. Glutathione S-transferase (GST) fusion proteins were prepared as described previously. For preparation of GST-free proteins, GST was cleaved from GST fusion proteins using PreScission Protease according to specifications from the manufacturer (Amerham Pharmacia Biotech, Tokyo). The purity of recombinant mouse VEGF-B186 and human bFGF was assessed by 10% SDS-PAGE and quantified by BCA protein assay reagent (Pierce, Rockford, U.S.A.). Its biological activity was confirmed using a collagen gel tissue culture assay.

Collagen Gel Tissue Culture  Pregnant female TNX+/+ or TNX−/− mice 9.5 d post coitus (E9.5) were sacrificed, and their embryos were removed and placed in a sterilized phosphate-buffered saline (PBS). Hearts of E9.5 embryos were collected. Collagen gel (final concentration, 3.0 mg/ml) was polymerized in 24-well culture plates according to the protocol established by the manufacturer (Nitta Gelatin Co. Ltd., Osaka). After the gel had solidified, serum-free culture medium (HAM F12 and DMEM in a 1:1 ratio) containing VEGF-A (100 ng/ml), VEGF-B (100 ng/ml) or bFGF (50 ng/ml) supplemented with penicillin (100 units/ml) and streptomycin (100 mg/ml) was added to the culture plates, and then the hearts of E9.5 embryos from TNX+/+ or TNX−/− mice were cultured on the collagen gel. Control explants (mock) were incubated in the same culture medium but in the absence of growth factors. The explants were cultured for 5 d, and the media were changed every 48 h.

Proliferation Area Measurement  During the incubation period, a sheath of proliferating cells formed around each explant. The proliferating cells were photographed using an Olympus IX70 inverted microscope containing an Olympus SC35 camera. This was followed by measurements using the NIH image Macintosh computer program available at http://rsb.info.nih.gov/nih-image/. At least 11 measurements were performed for each explant. Data were presented as the mean with standard deviation (mean ± S.D.). Statistical evaluation of the data was carried out using Student’s t-test.

RNA Purification and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)  Total RNA from the explants of E9.5 hearts was isolated as previously described. The RT-PCR conditions were used as the same as those described in our previous paper. For the detection of mouse VEGFR-1, the following primer sets were used: forward, 5′-ATCAAGACCTTCTGCAGCCGGCCTG-3′; reverse, 5′-TCCAGGTTAACCCGCTTCAGCAAG-3′. For the detection of mouse VEGFR-2, the following primer sets were used: forward, 5′-CCAGAAAGTAAAGGCCAAGC-3′; reverse, 5′-AGCAACATCTTCTCGTGATTTCTC-3′. For the detection of mouse TNX, the following primer sets were used: forward, 5′-ATGGCGACCTAGTGACACCCCCGTCTCA-3′; reverse, 5′-AAAGACACCCGGAGGCTCGACAGGC-3′.

RESULTS

We have already shown that VEGF-A as well as VEGF-B binds to TNX and that the association of VEGF-B with TNX enhances endothelial cell proliferation by measuring [3H]-thymidine incorporation. But we have not yet examined whether VEGF-A also increases the proliferation of endothelial cells when combined with TNX.

In this paper, we investigated the effect of TNX on cell proliferation by VEGF-A using embryonic hearts from wild-type (+/+) or TNX-deficient (−/−) mice on a collagen gel, since the collagen gel culture system is believed to mimic or closely reflect the in vivo environment. Recently, we generated TNX−/− mice by homologous recombination using ES cells. For the condition of presence or absence of TNX, we used wild-type (TNX+/+) or TNX−/− mice. TNX−/− mice were fertile and appeared healthy, with no gross abnormalities observed upon macroscopic inspection; however, we expected different responses to VEGF family members with regard to endothelial cell proliferation on the collagen gel between TNX+/+ and TNX−/− mice.

When E9.5 heart explants from wild-type mice were incubated in the presence of bFGF, the cell proliferation area was significantly greater than in the controls (mock) (Figs. 1A, B). Furthermore, since the control explants (mock) from both wild-type (TNX+/+) and TNX−/− mice showed the same extents of cell proliferation, indicating that endogenous growth factors secreted from the explants and their receptors are expressed to almost the same extents in both explants. These results indicate that this assay system is useful for the detection of exogenous growth factor activity. During the 5-d incubation period, mesenchymal cells proliferated from the explants onto the collagen substrate and formed a characteristic cobblestone monolayer. This continuous layer extended further from the explants with time due to the proliferative activities. Occasionally, elongated cell also emerged around the explants irrespective of the presence or absence of bFGF, VEGF-A165, or VEGF-B186 (Fig. 1A). It is known that most cells growing on the surface of collagen gel are epithelial cells forming an embryonic epicardial mesothelium, which differentiates into endothelial cells. By using this culture system, E9.5 heart explants from TNX+/+ or TNX−/− mice were incubated with VEGF-A165 or VEGF-B186 for 5 d. The addition of VEGF-A165 to TNX+/+ mouse explants led to a 1.5-fold greater cell proliferation than that in the case of addition of VEGF-A165 to TNX−/− mouse explants, whereas VEGF-B186 had no effect on cell proliferation in TNX+/+ mouse explants compared to that in the case of TNX−/− mouse explants (Figs. 1A, B) (see Discussion). To try to obtain evidence that the embryonic heart explants respond to VEGF-A165, leading to the cell proliferation, we examined whether the heart explants express VEGFR-1 and VEGFR-2 by RT-PCR. As expected, both VEGFR-1 and VEGFR-2 were expressed to the same extents in the heart explants from either TNX+/+ or TNX−/− mice (Fig. 1C). These results indicate that a VEGF family member, especially VEGF-A, together with TNX enhances proliferation of cells (progenitors of endothelial cells) compared to that in the case of a VEGF family member alone in an E9.5 heart explant cultured system.

DISCUSSION

In a previous study, we demonstrated that TNX in combination with VEGF-B increases endothelial cell proliferation. In the present study, we obtained evidence that the
proliferation of cells (progenitors of endothelial cells) out-growing from E9.5 heart explants is significantly increased by VEGF-A with TNX. These findings suggest that VEGF-A together with TNX is important for the control of endothelial cell proliferation in vivo.

A collagen gel culture system closely reflects the in vivo environment, but the specific cell responses and interactions in vivo and in vitro differ. Ratajska et al.\textsuperscript{16} demonstrated that the addition of VEGF-A to rat E14 atrial explants, but not to ventricles or whole hearts, stimulates the appearance of a cord-like structure. In contrast, treatment of rat E12 atrial and ventricular explants with VEGF-A did not induce cell proliferation, nor the appearance of cord-like structures. In this work, we used mouse E9.5 whole heart explants. Cord-like structures were not formed even during prolonged incubation either in the presence or absence of a VEGF family

Fig. 1. Effects of VEGF-A on Proliferation of Cells from E9.5 Heart Explants Cultured on Collagen Gels

(A) Phase contrast micrographs. E9.5 heart explants from wild-type (TNX\textsuperscript{+/-}) (a–d) or TNX-deficient (TNX\textsuperscript{-/-}) (e–h) mice were incubated for 5 d on collagen gels in the presence of 100 ng/ml VEGF-A\textsubscript{165} (b, f), 100 ng/ml VEGF-B\textsubscript{186} (c, g), and 50 ng/ml bFGF (d, h). ‘Mock’ (a, e) indicates control explants incubated with the same culture medium but without growth factors. Scale bar=1 mm. (B) Quantitation of VEGF-A-induced cell proliferation. The area of the cell monolayer proliferating from each explant was fringed with a white line as shown in (A). The area was calculated using the computer program NIH Image. Each point represents the mean±S.D. of at least 11 samples. Two asterisks: statistically significant difference against mock ($p<0.01$; $n=11$). One asterisk: statistically significant difference against mock ($p<0.05$; $n=11$). White star: statistically significant difference between TNX\textsuperscript{+/-} and TNX\textsuperscript{-/-} mice ($p<0.05$; $n=12$). Black star: statistically significant difference between TNX\textsuperscript{+/-} and TNX\textsuperscript{-/-} mice ($p<0.05$; $n=14$). (C) Expression of VEGFR-1 and VEGFR-2 in E9.5 hearts from TNX\textsuperscript{+/-} and TNX\textsuperscript{-/-} mice. RT-PCR analysis was performed with total RNA from E9.5 hearts of TNX\textsuperscript{+/-} and TNX\textsuperscript{-/-} mice. To confirm the absence of TNX expression in TNX\textsuperscript{-/-} mice, RT-PCR was also done using TNX-specific primers.
member. However, elongated cells occasionally appeared around the explants. The reason why a cord-like structure was not detected in the present study may be because of different embryonic stages of the explants and/or different experimental conditions from previous studies.16)

As for signaling pathway by VEGF-A, VEGFR-1 and VEGFR-2 generate the mitotic signals, whereas VEGF-B-elicited signals is mediated by VEGFR-1.13) In this paper, we demonstrated that VEGF-A together with TNX increases the cell proliferation from E9.5 heart explants more efficiently than in the absence of TNX. Although further study is necessary, we speculate that tyrosine phosphorylations of VEGFR-1/2 are occurred in TNX explants, whereas VEGF-B had no effect. This result regarding the effect of VEGF-B is not consistent with our previous results, namely, enhanced effect of VEGF-B was not detected in the present study may be because of different experimental system. It is known that VEGF-A-elicited signal transduction via VEGFR-2 is stronger than signaling events generated by VEGF-B via VEGFR-1.20) In this embryonic heart explant system, the heart explant by itself expresses much more unknown factors which affect its proliferation than the endothelial cell line. Thus, it is possible that in the heart explant system the effect of VEGF-B which has weak signal transduction on cell proliferation could not be detected, but strong effect of VEGF-A could be only detected.

Interestingly, as shown in Figs. 1A and B, bFGF together with TNX also stimulates cell proliferation from E9.5 heart explants compared to that in the case of bFGF alone. We have not yet clarified whether bFGF binds to TNX. However, if it binds to TNX, TNX might augment the functions of bFGF on cell proliferation as in the case of VEGF family with TNX.

In conclusion, we have revealed that in an in vitro cell cultured system TNX augments the effects of VEGF-A on endothelial cell proliferation. These findings provide an insight into the in vivo function of TNX in the regulation of development of blood vessels, such as vasculogenesis and angiogenesis.

REFERENCES