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Overexpression of Latent Transforming Growth Factor-β1 (TGF-β1) Binding Protein 1 (LTBP-1) in Association with TGF-β1 in Ovarian Carcinoma

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Using the differential display method, latent transforming growth factor-β1 (TGF-β1) binding protein 1 (LTBP-1) mRNA was identified as one of the enriched mRNAs in ovarian carcinoma tissues after isolation of genes responsible for the development of ovarian cancer. Semi-quantitative reverse transcription (RT)-PCR analysis showed that expression of LTBP-1 and TGF-β1 mRNAs was much higher in both serous and mucinous adenocarcinomas than in their benign counterparts, including serous and mucinous cystadenomas and cystadenomas of low malignant potential (LMPs). Immunohistochemical analysis demonstrated that only proliferating benign adenoma cells were immunoreactive for both LTBP-1 and TGF-β1 proteins. In contrast, most serous and mucinous adenocarcinoma cells and their surrounding stroma were intensely immunoreactive for LTBP-1 and TGF-β1. LTBP-1 and TGF-β1 proteins, and their complex forms were identified in ovarian carcinoma cell lines and in their culture media by western blot analysis, suggesting these products were produced in ovarian carcinoma cells. RT-PCR analysis demonstrated that LTBP-1L, one of the LTBP-1 transcripts that has a strong activity in targeting the latent form of TGF-β1 to extracellular matrix (ECM), was predominantly expressed in ovarian carcinomas. Taken together, the results suggest that upregulation of LTBP-1 in ovarian carcinoma cells may have an important role in distributing TGF-β1 in the stromal tissues surrounding carcinoma cells.

Key words: LTBP-1 — Overexpression — TGF-β1 — Ovarian carcinoma

Carcinogenesis is thought to be a multi-step process involving several genetic changes. Ovarian cancer is the most lethal gynecologic malignancy, and its overall 5-year survival rate is as low as 30%.[1)] Investigation of the genetic changes that occur in ovarian cancer may improve our understanding of the carcinogenic process in the ovary. Additionally, the results may be useful for establishing good diagnostic or prognostic markers for ovarian cancer. In this study, differential display analysis was used to isolate genes that were preferentially expressed in ovarian carcinomas as compared with their benign counterparts. Latent transforming growth factor (TGF)-β1 binding protein 1 (LTBP-1) mRNA was isolated as one of the mRNAs enriched in ovarian carcinomas.

TGF-β1 is secreted from cells as biologically inactive forms consisting of the mature growth factor associated with the N-terminal peptide, a latency associated peptide (LAP), and LTBP-1. LAP is non-covalently linked to TGF-β1 to form the small latent form of TGF-β1 (Fig. 1D). On the other hand, LTBP-1 was first purified as a protein bound to the small latent form of TGF-β1.[2–4)] LTBP-1 is disulfide-linked to LAP through the third eight-cysteine repeat, forming the large latent complex of TGF-β1 (Fig. 1D).[5, 6)] This large latent complex is more efficiently secreted than the small latent form of TGF-β1. After secretion LTBP-1 is known to target TGF-β1 to the extracellular matrix, which probably serves as a storage site, from which the growth factor can be activated effectively.

Generally, the expression of TGF-β1 inhibits the growth of most cells except mesenchymal cells, and pure cultures of epithelial and endothelial cells are particularly sensitive to TGF-β1.[7)] In cancer cells, expression of TGF-β1 in epithelia suppresses tumorigenesis.[8, 9)] However, after carcinoma cells lose their sensitivity to TGF-β1 growth inhibition, the presence of TGF-β1 may contribute to tumor progression by affecting tumor invasion or angiogenesis,[10, 11)] inducing the synthesis of other growth factors from stromal cells,[12)] and inhibiting immune responses.[13, 14)]

In the present study, we demonstrated high expression of *LTBP-1* and *TGF-β1* genes in ovarian cancer cells and their surrounding stromal tissues by semi-quantitative reverse transcription (RT)-PCR and immunohistochemical analysis. The expression pattern of latent TGF-β1 complexes and the activation mechanisms of TGF-βs vary in different organs.[15)] In particular, the mechanism of activa-

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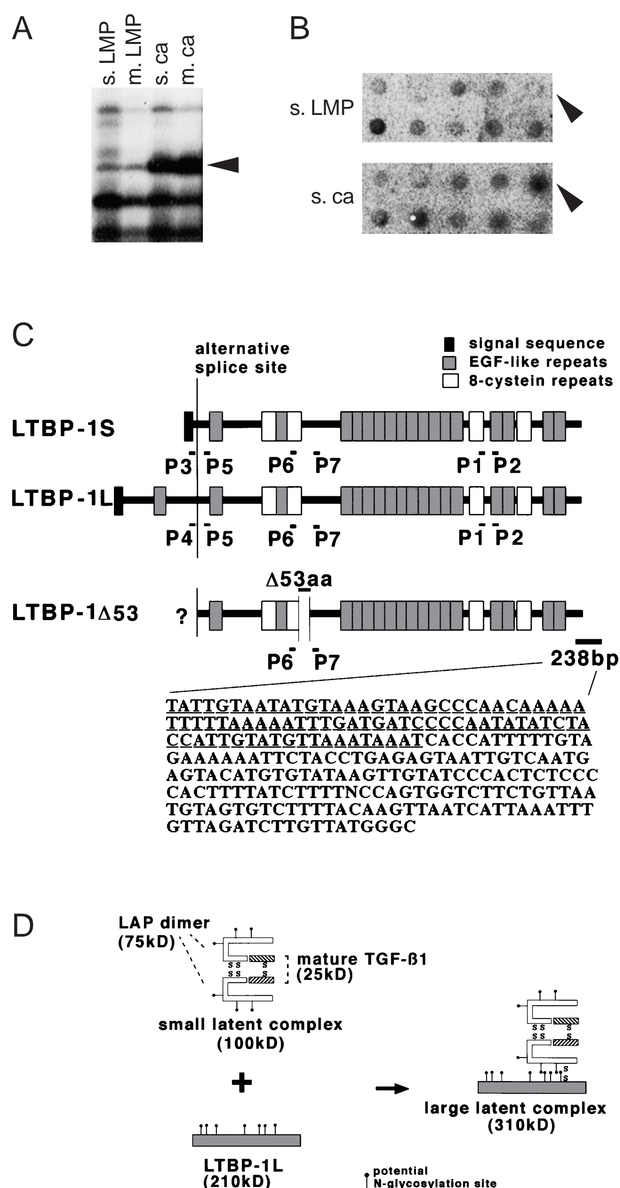

Fig. 1. A representative differential display autoradiogram using a tissue sample of serous LMP, mucinous LMP, serous adenocarcinoma and mucinous adenocarcinoma (A), a reverse northern analysis (B), a schema of the isolated LTBP-1 mRNA clone (C), and a schema of the structure of latent TGF-β1 and LTBP-1 (D). A and B: The arrowheads indicate the bands corresponding to LTBP-1 mRNA that was preferentially expressed in carcinomas. C: The protein structures of LTBP-1S, LTBP-1L and LTBP-Δ53, and all primers position (P1–7) are shown. A 238 bps cDNA clone was isolated by the differential display method. A part of sequence identical to that of human LTBP-1 mRNA clone (accession No. M34057) is underlined. P1 and P2 indicate the sense and antisense primers used to quantify LTBP-1. The sense primers P3 and P4 were used with a common antisense primer P5 for amplifying LTBP-1S and LTBP-1L transcripts, respectively. P6 and P7 were used to distinguish between splice variant LTBP-1Δ53 and the others.

tion of TGF-β1 and the function of LTBP-1 in ovarian tissue are not well understood. We discuss the role of upregulated LTBP-1 and its function associated with TGF-β1 in ovarian carcinoma tissues. This study raises many questions about the function of LTBP-1 and TGF-β1 in the carcinogenic process that require further investigation.

MATERIALS AND METHODS

Samples and RNA isolation Surgical ovarian tumor specimens, including serous and mucinous cystadenomas, cystadenomas of low malignant potential (LMPs), and carcinomas, were obtained at surgery after informed consent had been obtained, frozen immediately, and stored at −80°C. Of the 10 benign patients, 2 were postmenopausal and 8 were premenopausal women. Of the 8 premenopausal women, 5 were in the proliferative phase and 3 in the secretory phase of the menstrual cycle. Postmenopausal status was defined as 1 or more years since the last menstrual period. All the carcinomas were graded as worse than III according to the FIGO classification of ovarian cancer.[16] Total RNA was purified from these specimens by the guanidium isothiocyanate and cesium chloride method[17] and stored at −80°C. Ovarian surface epithelium of serous cystadenoma was taken as normal epithelium.

Cell culture HTOA cells derived from ovarian serous adenocarcinoma were purchased from Riken Cell Bank (Ibaraki) and maintained in HamF12 medium (Sigma, St. Louis, MO) containing 15% fetal bovine serum (JRH Bioscience, Lenexa, KS), 100 U/ml penicillin, and 100 μg/ml streptomycin. MCAS cells derived from ovarian mucinous adenocarcinoma purchased from HSRRB (JCRB, Osaka) were maintained in Eagle's minimum essential medium (MEM) (GIBCO/BRL, Rockville, MD) with 20% fetal bovine serum (JRH Bioscience), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Differential display The differential display was performed using an "RNAImage" kit (GenHunter Corp., Brookline, MA) according to the manufacturer's instructions. Briefly, a mixture of 0.2 μg of total RNA, 20 μM dNTPs, 0.2 μM anchored oligo dT primer (H-T_{11}A; 5′-AAGCTTTTTTTTTTTA-3′), and reverse transcription buffer in a 19-μl reaction volume was incubated at 65°C for 5 min, and then cooled at 30°C for 10 min. After addition of 1 μl of 100 U/μl MMLV, the mixture was incubated at 37°C for 50 min and then kept at 75°C for 5 min to inactivate the reverse transcriptase. Two-microliter aliquots of the reaction mixture were subjected to PCR amplification using the anchoring primer, H-T_{11}A, and an arbitrary 5′ primer AP55, (5′-AAGCTTACGTTAG-3′). The amplification reaction contained 2 μM each primer, 25 μM dNTPs, PCR buffer, [α-^{32}P]dCTP (0.25 μl/reaction, specific activity 110 TBq/mmol, Amersham Pharmacia

Biotech, Buckinghamshire, UK) in order to radiolabel the cDNA fragments, and *Taq* polymerase (TaKaRa, Shiga) in 20 μl of PCR buffer. The amplification profile was as follows: 94°C for 30 s, 40°C for 2 min, and 72°C for 30 s for 40 cycles followed by a final 5 min incubation at 72°C. Amplified ^{32}P-labeled cDNA samples were separated on 6% denaturing polyacrylamide gels, which were then exposed overnight to X-ray film. Candidate cDNA bands were cut out with a clean razor blade, soaked in 100 μl of TE, and then boiled for 15 min. The recovered cDNA samples were reamplified by PCR and purified by gel electrophoresis.

Reverse northern blot analysis, cloning, and sequencing The reamplified products were alkali-denatured and blotted onto nitrocellulose filter paper. Total RNA from either cystadenomas of low malignant potential or adenocarcinomas was reverse-transcribed in the presence of [α-^{32}P]CTP as described above, purified, and alkali-denatured. This ^{32}P-labeled cDNA was used as a probe and hybridized using HB-N[17] at 42°C overnight. The filter was washed twice with 2× SSC and 0.1% sodium dodecyl sulfate (SDS) at room temperature and twice with 0.1× SSC and 0.1% SDS at 65°C. After drying, the filters were analyzed with a BAS 2000 (Fujix, Tokyo). The confirmed preferentially expressed products were cloned into pGEMT vector (Promega, Madison, WI). The cloned cDNA was sequenced with an autosequencer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems, Norwalk, CT) using a dRhodamine Terminator Cycle Sequencing Kit (Applied Biosystems). The obtained sequence was used for a comparative computer search of the GenBank, EMBL, and DDBJ databases.

Semi-quantitative RT-PCR Semi-quantitative RT-PCR was performed as described elsewhere.[18] Briefly, a mixture of 1 μg of total RNA and 1 μl of oligo dT (10 pmol/μl) was incubated at 65°C for 5 min and cooled at 37°C for 5 min. After addition of 4 μl of 5× reverse transcriptase buffer (Toyobo, Osaka), 5 μl of 2 m*M* dNTP, 0.5 μl of RNase inhibitor (Toyobo), and 0.1 μl of 100 U/ml "ReverTra Ace" (Toyobo) to a total volume of 20 μl, the mixture was incubated at 50°C for 30 min and then at 75°C for 5 min to inactivate the reverse transcriptase. Two microliters of the cDNA mixture was subjected to PCR amplification as described above. The sequences of the primers were as follows: LTBP-1: 5′-CTGTATGGAGA-GGCCTGGGGCATG-3′ (sense)-P1, and 5′-GGATGCC-GCATTCCTCAGCCT-3′ (antisense)-P2, TGF-β1: 5′-CAGAAATACAGCAACAATTCCTGG-3′ (sense) and 5′-CAGTGTGTTATCCCTGCTGTC-3′ (antisense), and GAPDH: 5′-CATGGGGAAGGTGAAGGTCGGA-3′ (sense), 5′-TTGGCTCCCCCCTGCAAATGAG-3′ (antisense). The PCR cycle consisted of 1 min at 94°C, 1 min at 62, 65, or 50°C for LTBP-1, TGF-β1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively, and 1 min at 72°C. Amplifications for 20, 22, 24, 26, 28, 30, 32, 34, and 36 cycles were used to determine the number of cycles required for saturation. The number of cycles was chosen to be within the exponential region of the PCR reaction. Thirty cycles were used for the amplification of LTBP-1 and TGF-β1, and 25 cycles for GAPDH. Each PCR product was electrophoresed in a 2% agarose gel and stained with ethidium bromide. Photographs of the gels were converted to Macintosh-format PICT files. The intensity of each product was measured on a Macintosh computer using NIH Image ver. 1.55. Relative levels of LTBP-1 and TGF-β1 mRNAs were quantified by normalizing the intensity of the obtained PCR product to the signal intensity obtained for the GAPDH PCR product from the same RNA aliquot.

To confirm which isoform of LTBP-1 (S or L) was preferentially induced, PCR was performed using sense primers that distinguished the types (P3: 5′-CCAAGGCAA-GTTCATGGATACTAA-3′ for LTBP-1S and P4: 5′-CGT-GCTCAAGCCCAAGTACTTTC-3′ for LTBP-1L) and an antisense primer common to these isoforms (P5: 5′-TG-TCGGCAGCATGACCATTC-3′). To distinguish LTBP-1Δ53 from the no-deletion copy, the P6: 5′-CCTGTTACC-GACTTGTCAGT-3′ (sense), and P7: 5′-TGACCAGGC-TCAAGTTTGGT-3′ (antisense) primer set was used.

Immunohistochemistry Fifty-seven samples were immunohistochemically analyzed. Twenty of them were the same paraffin-embedded tissue samples used for RT-PCR analysis, and the other 37 were archived samples. Antibody against LTBP-1 (Ab39; rabbit polyclonal IgG) was kindly provided by Dr. Carl-Henrik Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden).[19] The affinity-purified rabbit polyclonal antibody against TGF-β1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Paraffin-embedded specimens were cut into 4 μm sections, mounted on coated glass slides, dewaxed in xylene, and rehydrated through graded ethanol solutions. The slides were washed in distilled water and then immersed in methanol with 0.3% H_2O_2 for 30 min to quench endogenous peroxidase. After incubation with normal goat serum for 20 min, tissue sections were incubated overnight at 4°C with the specific antibodies diluted 1:200 (anti-LTBP-1 antibody) and 1:250 (anti-TGF-β1 antibody) in phosphate-buffered saline (PBS) containing 1% normal goat serum. Immunoreaction was visualized using the ABC high-HRP Immunostaining Kit (Toyobo) and 0.05% (w/v) diaminobenzidine solution with 0.003% H_2O_2. The slides were counterstained in Mayer's hematoxylin.

Western blotting For preparation of cell lysates, essentially the method described by Nakajima *et al.*[20] was used. MCAS cells were cultured to confluence in 100 mm culture-dishes with FBS free medium. Conditioned medium was collected and concentrated 20-fold in an Amicon (30-

kD cut off; Millipore, Bedford, MA). The cells was washed with PBS and then homogenized in 20 m*M* Tris/HCl (pH 7.4), 150 m*M* NaCl, 1% NP-40 and 1% sodium deoxycholate. Supernatant (cell lysate fraction) was recovered by centrifugation and concentrated in the Amicon (30-kD cut off; Millipore). Protein concentration was measured with a Bio-Rad assay kit (Bio-Rad, Hercules, CA). The concentrated medium from 400 μl of original medium and 50 μg of total cell lysate were subjected to 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) under non-reducing condition. Electrophresed proteins were transferred to Immobilon-P (Millipore). The membrane was blocked with 20 m*M* Tris-buffered saline (TBS) (pH 7.4) containing 10% non-fat dry milk, then incubated with anti-LTBP-1 antibody (Ab39) at 1:100 dilution or with affinity-purified goat polyclonal anti-LAP antibody (R&D Systems, Minneapolis, MN) at 1:100 dilution. The membrane was incubated with the appropriate horseradish peroxidase-conjugated second antibody (DAKO, Kyoto) at 1:1000 dilution, and protein signals were detected by a chemiluminescence method using ECL western blotting detection reagents (Amersham, Buckinghamshire, UK).

Statistical analysis Differences in signal intensities of LTBP-1 and TGF-β1 expression in different groups were evaluated by using Mann-Whitney's *U* test.

RESULTS

Identification of LTBP-1 mRNA from ovarian cancer To identify certain genes expressed differently in ovarian cancers than in benign counterparts, differential display analysis was performed using a tissue sample of serous cystadenoma of low malignant potential (serous LMP), mucinous LMP, serous adenocarcinoma and mucinous adenocarcinoma (Fig. 1A). Of the 36 candidate bands that were preferentially expressed in carcinomas, whether serous or mucinous, 24 bands could be reamplified. Subsequently, 6 of the 24 bands were cloned and sequenced because their preferential expression was confirmed by reverse northern blot analysis (Fig. 1B). Sequence analysis revealed that one was novel, two had homology with expressed sequence tags (ESTs), and three had homology with genes registered in GenBank databases. Although 2 of 3 were mitochondrial, one was identical to the 3′ non-coding region of TGF-β1 binding protein 1 (LTBP-1) mRNA (Fig. 1C). TGF-β1 is known to control cell proliferation and differentiation[21] and LTBP-1 plays a critical role in the secretion and activation of TGF-β1.[19] It is conceivable that regulation of these genes is associated with the development of ovarian cancer. Thus, we quantified expression levels and examined the localization of TGF-β1 and LTBP-1 in cystadenomas, cystadenomas of LMPs, adenocarcinomas, and two cell lines derived from ovarian carcinomas.

Quantification of LTBP-1 and TGF-β1 mRNA using RT-PCR in ovarian tissues Since insufficient mRNA was obtained from benign cystadenoma and LMP tissues, we could not compare the expression levels between benign and malignant tissues by northern blot analysis. Therefore, we examined expression of *LTBP-1* and *TGF-β1* genes in different tumors by RT-PCR analysis. Neither LTBP-1 nor TGF-β1 mRNA was detected in serous cystadenoma cells (Fig. 2A, lane 1). In mucinous cystadenomas, LTBP-1 mRNA was not detected, while faint bands for TGF-β1 mRNA were observed (Fig. 2A, lane 2). In both serous and mucinous LMPs, faint bands for

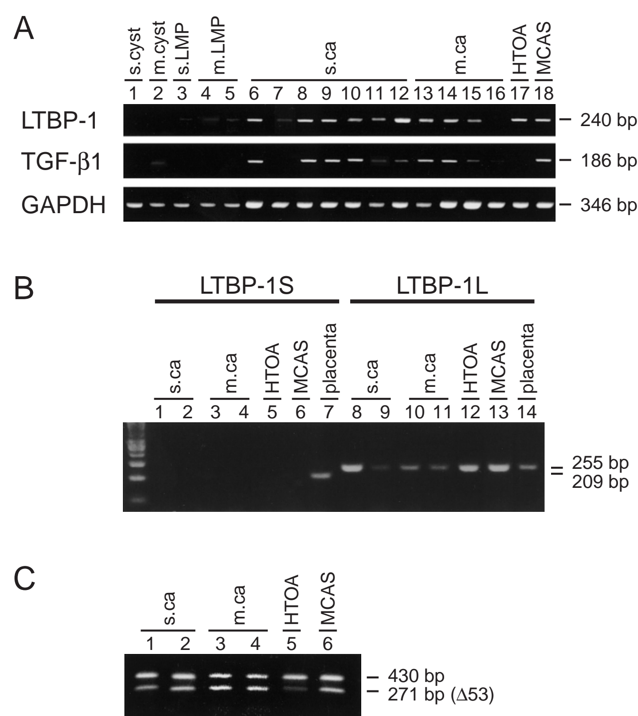

Fig. 2. A: Ethidium bromide staining of the RT-PCR products of LTBP-1, TGF-β1 and GAPDH. Lane 1, serous cystadenoma; 2, mucinous cystadenoma; 3, serous LMP; 4–5, mucinous LMPs; 6–12, serous adenocarcinoma; 13–16, mucinous adenocarcinoma; 17, HTOA; and 18, MCAS. B: The RT-PCR products for LTBP-1S and LTBP-1L using P3 and P5, P4 and P5 primers, respectively. Lanes 1 and 8, serous adenocarcinoma, same as lane 12 in A; lanes 2 and 9, serous adenocarcinoma, same as lane 11; lanes 3 and 10, mucinous adenocarcinoma, same as lane 13; lanes 4 and 11, mucinous adenocarcinoma same as lane 14; lanes 5 and 12, HTOA; lanes 6 and 13, MCAS; lanes 7 and 14, placenta as a positive control. The 209 and 255-bp bands correspond to LTBP-1S and LTBP-1L, respectively. C: The RT-PCR products for Δ53 (271 bp) and the other LTBP-1 (430 bp) transcript using P6 and P7 primer. Lanes 1 and 2, serous adenocarcinoma; 3 and 4, mucinous adenocarcinoma; 5, HTOA; and 6, MCAS.

LTBP-1 mRNA were detected, while no TGF-β1 mRNA was observed (Fig. 2A, lanes 3–5). On the other hand, significantly high amounts of LTBP-1 mRNA were detected in all serous adenocarcinoma tissues. TGF-β1 mRNA was expressed at levels as high as LTBP-1 in 6 of 7 serous adenocarcinomas (Fig. 2A, lanes 6–12), and in 3 of 4 mucinous adenocarcinomas (Fig. 2A, lanes 13–16). One serous adenocarcinoma displayed LTBP-1 only (Fig. 2A, lane 7). High expression of LTBP-1 mRNA was also observed in two ovarian carcinoma cell lines, HTOA (derived from human serous adenocarcinoma), and MCAS (derived from human mucinous adenocarcinoma). TGF-β1 mRNA, however, was detected only in MCAS (Fig. 2A, lanes 17 and 18).

It has been reported that two mRNAs encoding LTBP-1S or LTBP-1L are transcribed from different promoters.[22] LTBP-1L (long) is a splice variant, which contains an amino-terminal extension of 346 amino acids not found in the smaller LTBP-1S (small) isoform. We chose primer pairs to distinguish between LTBP-1S and LTBP-1L mRNA in RT-PCR (Fig. 2B). The LTBP-1L transcript (255 bps) was detected in both serous and mucinous adenocarcinoma tissues and the cell lines (Fig. 2B), whereas the LTBP-1S (209 bps) was not detected. The latter transcript was detected only in placental tissue. It is reported that another splicing variant for LTBP-1, called Δ53 exists, although its functional role is unknown.[23] When we used primers to detect this splice variant, the amount of Δ53 was equivalent to the transcript for LTBP-1L (Fig. 2C). From these findings, high expression of the *LTBP-1* gene in ovarian carcinomas may result from upregulation of the *LTBP-1L* and *LTBP-1 Δ53* genes, but not *LTBP-1S*.

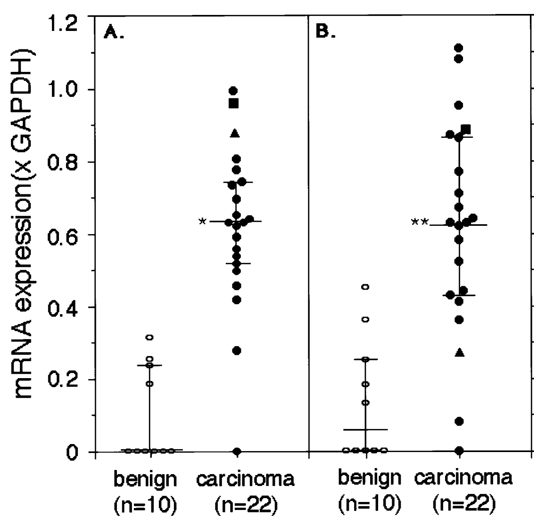

Fig. 3. The expression of LTBP-1 (A) and TGF-β1 (B) mRNA in benign ovarian tumors and carcinomas. Expression levels for LTBP-1 and TGF-β1 mRNAs were calculated from the ratios of LTBP-1 or TGF-β1 to GAPDH mRNA signal intensities. Benign tumors include four cystadenomas and ten LMPs. Carcinomas include twelve serous and ten mucinous adenocarcinomas. The white circles and the black circles represent values of benign tumors and carcinomas, respectively. The black triangles and the black squares represent values of HTOA and MCAS, respectively. Bars indicate the medians [25%, 75%] in each group. Mann-Whitney's *U* test: * $P<0.0001$, ** $P=0.0003$.

Then LTBP-1 and TGF-β1 RT-PCR products were quantified, using GAPDH as an internal control, in ten benign tumors, including four cystadenomas and six LMPs, and 22 adenocarcinomas (Fig. 3). LTBP-1 and TGF-β1 expression levels were significantly higher in carcinomas than benign tumors (Mann-Whitney's *U* test, $P<0.0001$ and $P=0.0003$, respectively). The magnitude of expression of LTBP-1 and TGF-β1 did not differ between serous ($n=12$) and mucinous ($n=10$) adenocarcinomas (Mann-Whitney's *U* test, data not shown). To study whether *LTBP-1* and *TGF-β1* genes were simultaneously upregulated or not, transcriptional levels of *LTBP-1* and *TGF-β1* genes were compared in each specimen (Fig. 4). Although no correlation was observed in benign tumors (correlation coefficient: $r=-0.179$), *TGF-β1* gene expression was associated with that of LTBP-1 in carcinomas (correlation coefficient: $r=0.787$, $P<0.0001$). If two carcinoma samples without expression of LTBP-1 or TGF-β1 were omitted, the association would be even more significant.

Immunohistochemistry of LTBP-1 and TGF-β1 in ovarian tumor tissues Immunohistochemical analysis showed that LTBP-1 and TGF-β1 were rarely expressed in normal ovarian surface epithelia (Fig. 5, A and B). In serous cystadenoma and LMP, both LTBP-1 and TGF-β1 were expressed in the cilia and cytoplasm of a few adenoma cells (Fig. 5, C and D); such signals were also observed in the basement membrane of mucinous epithelium (Fig. 5, E and F). In mucinous LMP, LTBP-1 and TGF-β1 signals were observed in the cytoplasm facing the basement membrane and the basement membrane itself (Fig. 5, G and H). Interestingly, most of these LTBP-1 and TGF-β1 signals were observed in dividing epithelial cells in serous adenoma (Fig. 5, C and D) or in proliferating cells on the papillary project in mucinous cystadenoma (Fig. 5, E and F). In contrast, LTBP-1 and TGF-β1 were weakly expressed in the stromal tissue underlying epithelium in both serous and mucinous cystadenoma and their LMPs. Strong expression of both LTBP-1 and TGF-β1 proteins was observed in both serous (Fig. 5, I and J) and mucinous (Fig. 5, K and L) adenocarcinoma cells. Interestingly, intense signals for both LTBP-1 and TGF-β1 were also observed in the extracellular matrix (ECM) of the stromal tissues surrounding cancer cell nests (Fig. 5, I and L).

To compare expression levels of LTBP-1 and TGF-β1 proteins in different specimens, the ratio of the epithelial

cells expressing these proteins in total epithelial cells was calculated (Fig. 6). Twenty-two benign tumor samples containing ten cystadenomas and twelve LMPs, and 35 carcinoma samples containing 15 serous and 18 mucinous adenocarcinomas and 2 cell lines were analyzed. Cells positive for LTBP-1 and TGF-β1 proteins were more frequent in carcinomas than in benign tumors (Mann-Whitney's U test, $P<0.0001$). Percentages of positive cells for LTBP-1 and TGF-β1 did not differ between serous and mucinous adenocarcinomas (Mann-Whitney's U test, data not shown).

Expression of LTBP-1 and TGF-β1 proteins in ovarian carcinoma cell lines We have demonstrated abundant expression of LTBP-1 and TGF-β1 proteins in ovarian carcinoma tissues. These proteins were localized in both carcinoma cells and stromal tissues. To see whether these proteins were synthesized in carcinoma cells, we exam-

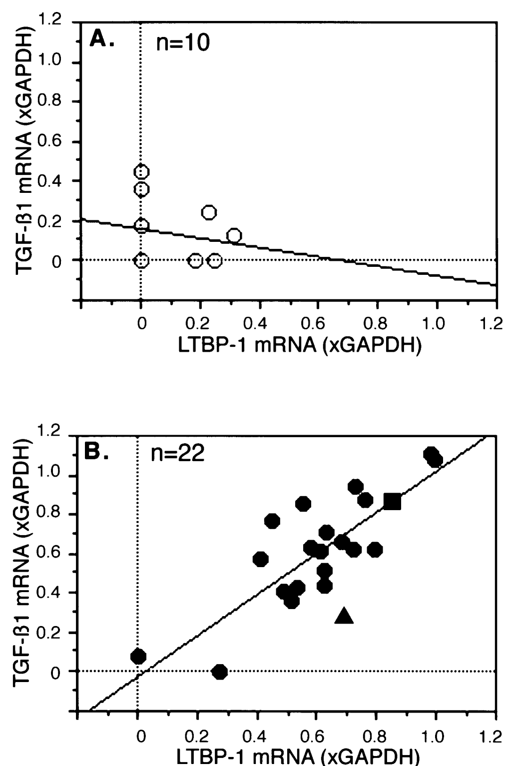

Fig. 4. The correlation in expression levels of LTBP-1 and TGF-β1 mRNA between benign ovarian tumors (A) and carcinomas (B). Each plot represents the ratios of LTBP-1 and TGF-β1 mRNA to GAPDH mRNA. (A) Regression line: TGF-β1= −0.234×LTBP-1+0.16; correlation coefficient, $r=-0.179$. (B) Regression line: TGF-β1=1.048×LTBP-1−0.026; correlation coefficient, $r=0.787$, $P<0.0001$. The white circles and the black circles represent values of benign tumors and carcinomas, respectively. The black triangles and the black squares represent values of HTOA and MCAS, respectively.

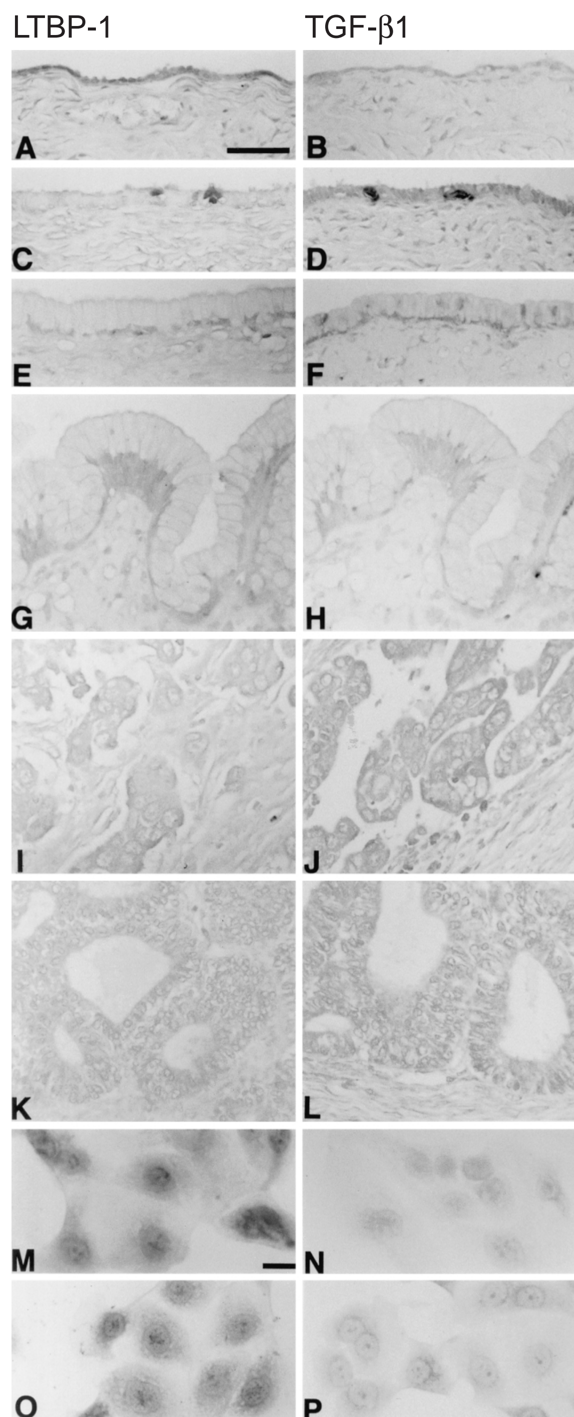

Fig. 5. Immunohistochemical staining of LTBP-1 and TGF-β1 in different ovarian tumors. A and B, normal ovarian surface epithelia; C and D, serous cystadenoma (corresponding to Fig. 2A lane 1); E and F, mucinous cystadenoma (lane 2); G and H, mucinous LMP (lane 5); I and J, serous adenocarcinoma (lane 8); K and L, mucinous adenocarcinoma (lane 14); M and N, HTOA; O and P, MCAS. A, C, E, G, I, K, M and O, LTBP-1 immunoreactivity and B, D, F, H, J, L, N and P, TGF-β1 immunoreactivity. The bars indicate 40 μm (A–L), and 20 μm (M–P).

ined expression of LTBP-1 and TGF-β1 proteins in monolayer cultures of ovarian carcinoma cell lines, HTOA and MCAS. In immunohistochemical analysis, LTBP-1 was expressed throughout carcinoma cells, including the nuclei of both cell lines (Fig. 5, M and O), whereas TGF-β1 protein was strongly expressed in the perinuclear cytoplasm (Fig. 5, N and P). When western blot analysis was performed in the cell lysate and culture medium of MCAS cells, both LTBP-1 and LAP antibodies detected a 310 kD band corresponding to large latent complex (Fig. 7). The LTBP-1 antibody detected broad protein bands from 230 to 130 kD in the culture medium. Since these bands did not react to anti-LAP antibody, they correspond to LTBP-1 protein. This broad band pattern is due to protein glycosylation of LTBP-1, as reported before.[4] A 150 kD band in the medium fraction which reacted to anti-LAP antibody is unidentified, but it may be due to modification of the latent TGF-β1 complex. In the cell lysate, anti-LAP antibody gave a very faint broad band around 100 kD in the original film, although it is not apparent in Fig. 7. This band corresponds to small latent TGF-β1 complex.

DISCUSSION

The differential display analysis showed that LTBP-1 mRNA was one of the enriched mRNAs in ovarian carcinomas, when compared with benign adenomas. Using semi-quantitative RT-PCR, we confirmed the high expression of LTBP-1 mRNA in serous and mucinous adenocarcinomas, while no such expression was observed in cystadenomas and cystadenomas of LMPs. Semi-quantitative RT-PCR also showed higher expression of TGF-β1 in ovarian carcinomas than in their benign counterparts. These findings were also seen in the immunohistochemical analysis, suggesting that the transcriptional upregulation of *LTBP-1* and *TGF-β1* genes contributes to the high levels of their protein expression in ovarian carcinoma tissues.

Other authors have already noted high expression of TGF-β1 in a variety of cancer cells.[24–28] It was also reported that TGF-β1 expression was slightly increased in ovarian cancer, whereas expression of LTBP-1 was decreased in Swedish women.[29] In the present study, LTBP-1 was upregulated in both serous and mucinous adenocarcinomas. We cannot explain the discrepant results for LTBP-1. In the previous study, expression of LTBP-1 was examined by an immunohistochemical method and *in situ* hybridization. The expression of mRNA for LTBP-1 was confirmed in ovarian carcinoma tissues in our report. Different procedures and different histological types of ovarian carcinomas examined may explain the disparate results. Transcriptional regulation of *LTBP-1* and *TGF-β1* genes may vary in different histological types of ovarian cancer.

In immunohistochemical analysis, both LTBP-1 and TGF-β1 proteins were rarely expressed in normal ovarian

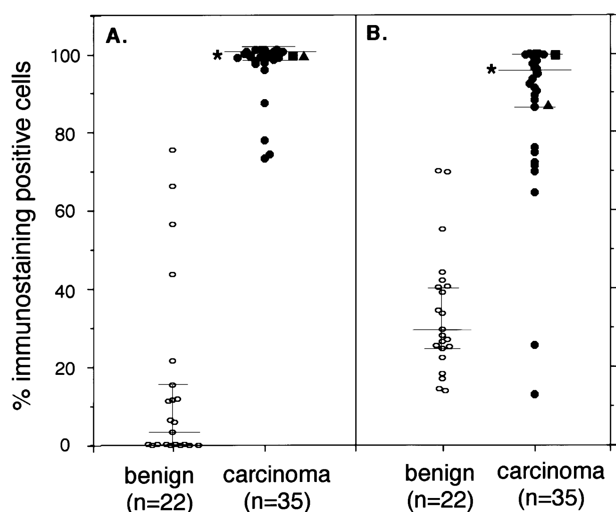

Fig. 6. Protein expression of LTBP-1 (A) and TGF-β1 (B) in benign ovarian tumors and carcinomas by immunohistochemical staining. Values represent percentages of positive cells for LTBP-1 and TGF-β1 in all epithelial cells. Benign ovarian tumors including cystadenomas and LMPs. The white circles and the black circles represent values of benign tumors and carcinomas, respectively. The black triangles and the black squares represent values of HTOA and MCAS, respectively. Bars indicate the medians [25%, 75%] in each group. * $P<0.0001$.

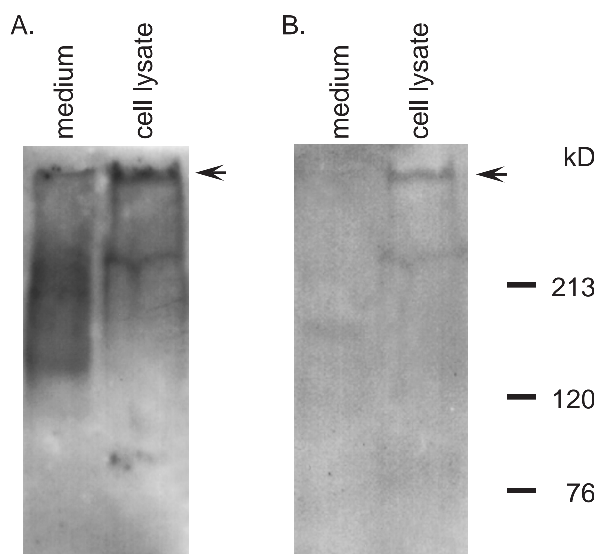

Fig. 7. Western blotting analysis using anti-LTBP-1 (A) and anti-LAP (B) antibody under nonreducing conditions. Lane 1, the conditioned medium of MCAS cells; 2, the cell lysate. The arrows at the 310 kD band indicate the large latent complex.

surface epithelia, but they were expressed in a few dividing epithelial cells in cystadenoma (Fig. 2, C and D) and LMP (Fig. 2, E and F). The proliferation of epithelial cells may be associated with the localization of LTBP-1 and TGF-β1 in benign tumors. TGF-β1 inhibits cell growth in normal tissues and in early cancers.[30, 31] It is surmised that the latent TGF-β1 released from the ECM first attaches to the epithelial surface, where it is activated by proteolysis and the signal is transduced to the cell interior after binding with the receptor.[21] In the present study, both LTBP-1 and TGF-β1 protein signals were restricted to the basement membrane and the proximal part of the cytoplasm of benign adenoma cells. These signals may be due to the latent TGF-β1 and some activated TGF-β1 transducing the growth-inhibitory signals to the proliferating adenoma cells.

In the present study, this coordinated localization pattern was not observed in carcinoma tissues. The LTBP-1 and TGF-β1 protein signals were observed throughout the entire cell of almost all carcinoma cells. These findings may suggest that carcinoma cells overcame the growth inhibitory effect of TGF-β1. Impairment of the signal transduction of TGF-β1 by mutation or deletion of *smad* genes has been reported in many cancers including ovarian cancer.[32] We should investigate the correlation between such gene abnormalities and overexpression of TGF-β1 in ovarian cancer cells. When we compared the expression levels of LTBP-1 and TGF-β1 proteins between an ovarian carcinoma cell line and carcinoma cells of clinical samples, we noticed that expression of TGF-β1 was lower in the cultured cells (Fig. 5, N and P) than in the clinical samples (Fig. 5, J and L). There may be some cross-talk between epithelial cells and stromal cells.

We demonstrated that high expression of LTBP-1 mRNA was predominantly due to upregulation of LTBP-1L mRNA rather than LTBP-1S in most ovarian carcinomas. The LTBP-1S promoter contains a TGF-β1 inhibitory element (TIE), whereas the LTBP-1L promoter does not.[22] Therefore, it is possible that upregulated TGF-β1 in ovarian cancer in turn down-regulates LTBP-1S expression through this mechanism. It is known that LTBP-1L protein can target the latent TGF-β1 complex to ECM more efficiently than LTBP-1S.[33] One epidermal growth factor (EGF)-like repeat at the N-terminal of LTBP-1L may be important for this tight association. When we compared the transcription levels of LTBP-1L and TGF-β1 (Fig. 4), expression of the two genes appeared to be closely associated. This finding suggests that these genes may share the same transcriptional machinery and such coordinated expression may be programmed for effective action of TGF-β1.

In western blot analysis using a mucinous adenocarcinoma cell line, MCAS, the large latent TGF-β1 complex was detected in the soluble fraction of the cell lysate. However, these forms were reduced in the culture medium, suggesting that the large latent complex may be unstable in the culture medium after secretion. Anti-LAP antibody could detect the large latent complex and a faint band of the small latent complex in the cell lysate. All these findings suggest that ovarian carcinoma cells are able to produce LTBP-1L, TGF-β1 and their complex forms. Since HTOA cell line grew very slowly, we could not perform western blot analysis for HTOA.

TGF-βs are multifunctional proteins, and there is debate as to whether they inhibit or promote cancer in humans. TGF-βs stimulate the growth of mesenchymal cells and angiogenesis,[11, 34] resulting in production of ECM in the stromal tissue of many organs.[35, 36] This stimulatory action of TGF-βs may be represented in the present finding of a strong stromal reaction observed in many ovarian carcinoma tissues. In contrast, TGF-βs also inhibit the growth of many cells, such as epithelial, endothelial, hematopoietic, and immune cells.[7] At the moment, we do not know whether the localization of TGF-β1 and LTBP-1 around carcinoma cells has a stimulatory or inhibitory effect on the carcinoma cells. It is reasonable to consider that the inhibitory function of TGF-β1 may be directed to the immune cells surrounding ovarian carcinoma cells. We have demonstrated upregulation of LTBP-1 in ovarian carcinoma tissues for the first time in the present study. The upregulated LTBP-1 appears to facilitate more efficient delivery of TGF-β1, which may serve as a gatekeeper against certain cell types. We have not yet elucidated how *LTBP-1* and *TGF-β1* genes are upregulated or how they function in the carcinogenic processes of ovarian epithelial cells. Further studies are needed to answer these questions.

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