Jpn. J. Cancer Res. **90**, 308–319, March 1999

High-molecular-weight Fibronectin Synthesized by Adenoid Cystic Carcinoma Cells of Salivary Gland Origin

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To understand the morphogenesis of characteristic cribriform structures and the frequent invasion of salivary adenoid cystic carcinomas (ACC) along such basement membrane-rich structures as peripheral nerves, we have isolated fibronectin (FN) from the culture media of ACC3 cells established from a parotid ACC and characterized its glycosylation and alternative splicing status. FN isolated from ACC3 cells (ACC-FN) showed a molecular mass of 315 kDa in SDS-PAGE and was less heterogeneous and larger than plasma FN (pFN) or FNs from other cell sources. Differential enzymatic treatments of immunoprecipitated ACC-FN with neuraminidase, peptide-N-glycosidase F and endo-α-N-acetylgalactosaminidase revealed that ACC-FN was composed of a polypeptide chain of 270 kDa, with 10 kDa each of N-linked and O-linked oligosaccharide chains. Reverse transcription polymerase chain reaction (RT-PCR), *in-situ* hybridization, and immunofluorescence studies showed that most ACC-FNs contained ED-A, ED-B and IIICS regions in the molecules. This alternative splicing status of ACC-FN seemed to contribute to its less heterogeneous and larger molecular form. Cell attachment assay demonstrated that ACC-FN was more potent than pFN in adhesion of ACC3 cells. The results indicated that ACC-FN may function as a substrate for attachment of ACC3 cells, or that ACC3 cells trap and retain ACC-FN in their pericellular space. This isoform of FN may play an important role in the mode of invasion of ACC and the formation of stromal pseudocysts in the characteristic cribriform structure of ACC.

Key words: Fibronectin — Adenoid cystic carcinoma — Extracellular matrix — Alternative splicing — Oligosaccharide

FN is a multifunctional glycoprotein present in the extracellular matrix and in various body fluids including plasma. It functions as a substrate for cell adhesion and spreading,[1)] thereby promoting cell migration during embryonic morphogenesis,[2)] wound healing,[3)] and tumor progression.[4)] These biological functions are based on the affinity of FN for cell surface molecules such as integrins and a number of extracellular matrix macromolecules, including collagen and HSPG.[5)] The binding characteristics of FN are related to its multiple domain structure. Further, there are structurally and functionally different isoforms of FN which result from alternative splicing of three regions, ED-A, ED-B and IIICS, of the FN primary transcript.[6, 7)] The ED-A and ED-B segments are included in or excluded from the mature mRNAs by exon skipping in a spatiotemporally and oncodevelopmentally defined manner,[8)] although the functions of these alternatively spliced segments are poorly understood. FNs isolated from plasma, various tissues and cells in culture are also posttranslationally modified with N-linked[9)] and O-linked[10)] oligosaccharide chains. Differences in oligosaccharide modification are likely to produce differences in properties such as solubility, sensitivity to protease digestion,[11)] and binding activity to other extracellular matrix molecules.[12)] It is hence supposed that the biological properties of cancer cells are also altered by the modification of oligosaccharide chains on FNs.

Adenoid cystic carcinoma, arising in the human salivary gland, is histopathologically characterized by cribriform structures which are formed by pseudocystic spaces with retention of basement membrane-associated molecules, especially FN and HSPG.[13, 14)] We have shown immunohistochemically and immunocytochemically that these extracellular molecules including FN are synthesized and secreted *in vitro* by ACC3 cells, which were

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The abbreviations used are: ACC, adenoid cystic carcinoma; CBB, Coomassie brilliant blue; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FN, fibronectin; HSPG, heparan sulfate proteoglycan; MEM, minimum essential medium; PBS, phosphate-buffered saline; pFN, plasma FN; PNGase, peptide-N-glycosidase; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SSC, salt sodium citrate.

established from a human adenoid cystic carcinoma.[15] Based on these data, we have suggested that the characteristic histology of adenoid cystic carcinoma results from the overproduction of basement membrane molecules by tumor cells, and that its frequent invasion along peripheral nervous tissues and metastasis on the hematogenous route rather than the lymphatic one reflects by the affinity of the tumor cells for basement membrane molecules, because vascular vessels and peripheral nerves are rich in basement membranes. We have further shown the oversynthesis of HSPG by this carcinoma cell line *in vitro* and characterized the molecule biochemically.[16] However, FN biosynthesized by ACC3 cells has not been well characterized.

In this study, we isolated FN from the culture of ACC3 cells (ACC-FN), and characterized its biosynthetic properties including its modes of alternative splicing and glycosylation. We further discuss the possible participation of ACC-FN in the characteristic histology and clinical behaviors of adenoid cystic carcinoma.

MATERIALS AND METHODS

Cell culture ACC3 cells, established from an adenoid cystic carcinoma of the human parotid gland, were cultured in RPMI-1640 medium (Nissui, Tokyo) with 1% glutamine (Nissui) as previously described.[15] MK-1 and ZK-1 cells from squamous cell carcinomas of the oral mucosa were cultured in DMEM (Gibco BRL, Grand Island, NY) as previously described.[17, 18] Human fibroblasts were isolated, with parental consent, from the stromal tissues of a melanotic neuroectodermal tumor of infancy arising in the mandibular bone of a 1-month-old boy, and were maintained in the same medium. All culture media contained 10% FCS (ICN Pharmaceuticals Inc., Costa Mesa, CA), 50 μg/ml streptomycin and 50 IU/ml penicillin G. Cells were incubated at 37°C in a humidified 5% CO_2/95% air atmosphere.

Purification of fibronectin Fibronectin produced by ACC3 was purified by gelatin affinity chromatography, according to the method of Sekiguchi *et al*.[19] ACC3 cells were plated at a concentration of 5×10^5 cells in 50 ml of the medium in a 15 cm plastic dish. When ACC3 cells became subconfluent 6 days after plating, the cells were washed and further incubated in RPMI-1640 lacking FCS for 24 h. The conditioned media were applied to a gelatin-Sepharose column (1.2×5 cm, Pharmacia Biotech, Uppsala, Sweden). Bound materials were eluted and concentrated, and fibronectin (ACC-FN) was identified on SDS-PAGE. The protein concentration of purified ACC-FN was estimated by using an image analysis system as described in the following section. pFN was purified from human plasma as described previously.[15]

Antibodies Antisera to ACC-FN purified as mentioned above were raised in rabbits by injections of 12 μg of protein and complete Freund's adjuvant into the bilateral popliteal lymph nodes, followed by two booster injections with 6 μg each of the antigen subcutaneously at 2-week intervals. The rabbits were bled 10 days after the last injection. Mouse monoclonal antibody against the ED-A (IST-9)[20] and against the ED-B (BC-1)[21] regions of human FN were kindly provided by Dr. Sekiguchi (Osaka University Institute for Protein Research, Osaka). Polyclonal antibodies against human pFN were raised in rabbits as described elsewhere.[15]

Immunoassay The specificity and the titer of the antibodies were tested by direct or competition ELISA techniques as described elesewhere.[22] For the competition, titer-matched anti-ACC-FN and anti-pFN were preincubated with serial dilutions (0.01−256 μg/ml) of pFN before addition to plates coated with pFN. *o*-Phenylenediamine (Nacalai Tesque, Inc., Kyoto) was used for color development for peroxidase-conjugated secondary antibodies. Absorbance at 492 nm was measured by a Dynatech NR-700 microplate reader (Dynatech Laboratories, Inc., Chantilly, VA).

Cell labeling and pulse-chase experiments Labeling experiments were performed as described elesewhere.[16] Cells were preincubated with methionine-free minimum essential medium (MEM) for 1 h and incubated in fresh MEM with 25 μCi of [^{35}S]methionine for 3 h. For pulse-chase experiments, the cells were pulse-labeled with 50 μCi of [^{35}S]methionine for 30 min and chased in the complete MEM for 30 min, 1 h, 2 h and 3 h. After removal of the media, the cells were lysed, and both the cell lysates and media were centrifuged at 15,000*g* for 10 min. The resultant supernatants were subjected to immunoprecipitation.

Immunoprecipitation The cell lysates and media were immunoprecipitated as described before.[16] The precleared lysates and media were incubated with the antibodies overnight, and immune complexes were isolated with protein A-Sepharose (Pharmacia Biotech). Immunoisolated materials were dissolved in Laemmli's sample buffer, boiled for 5 min, and centrifuged at 10,000*g* for 5 min to remove beads. The supernatants were analyzed by SDS-PAGE.

Enzyme digestion All enzymatic digestions were performed at 37°C for 10 h in the presence of the proteinase inhibitor mixture described above. Neuraminidase (Seikagaku Kogyo, Tokyo) digestion was carried out in 75 m*M* acetate buffer (pH 6.5) containing 10 m*M* and 320 mU/ml of enzyme. Digestion with peptide-N-glycosidase F (PNGase F, New England Biolabs Inc., Berrerly, MA) was carried out in 50 m*M* Tris-HCl (pH 7.5) containing 0.4% SDS, 1% Nonidet P-40 and 0.15 U/ml enzyme. Digestion with endo-α-N-acetylgalactosaminidase (Seikagaku Kogyo) was done in 200 m*M* citrate buffer (pH

4.5) and 200 mU/ml enzyme. All enzyme-digested materials were dissolved in Laemmli's sample buffer and processed as described above.

To prevent addition of N-linked oligosaccharide to ACC-FN, cells were preincubated with methionine-free MEM containing 2 μg/ml of tunicamycin (Sigma Chemical Co., St. Louis, MO) for 1 h and then incubated in fresh MEM with 50–100 μCi of [^{35}S]methionine containing 2 μg/ml tunicamycin for 3 h.

SDS-PAGE SDS-PAGE was done on 5% polyacrylamide slab gels with or without 2.5% 2-β-mercaptoethanol, according to Laemmli.[23] Gels were stained with CBB and then air-dried. Dried gels were fluorographed on X-ray film (Hyperfilm-MPTM, Amersham International plc, Buckinghamshire, England). Apparent molecular weights were determined by coelectrophoresis of marker proteins.

Quantitative analysis CBB-stained bands after SDS-PAGE were scanned by a Sharp flatbed scanner JX-320M (Sharp Corp., Tokyo) and the data were saved on a personal computer. The scanned images were analyzed and the relative amounts of FN bands were quantitated by using NIH Image, an image-processing and analysis program.

RT-PCR amplification of alternatively spliced FN mRNAs Total RNA was isolated from confluent cultures of ACC3 cells using the ISOGEN system (Nippon Gene Co., Ltd., Tokyo). cDNAs were synthesized from the RNA with the SuperScript Preamplification System (Gibco BRL). Following the RT, PCR was carried out in an Astec thermal cycler PC-800 (Astec Co., Ltd., Fukuoka) as follows. Reaction products of the RT were diluted with 1× PCR buffer [50 m*M* KCl, 10 m*M* Tris-HCl (pH 8.5), 1.5 m*M*, 0.01% Triton X-100] to a final volume of 50 μl, which contained 100 ng each of a forward oligonucleotide primer and a reverse primer, additional dNTPs (final concentration of 0.2 m*M*), and 2.5 units of *Taq* DNA polymerase (Takara Shuzo Co., Ltd., Kyoto). Oligonucleotide primers in the exons flanking ED-A, ED-B and IIICS regions, which are alternatively spliced, were synthesized according to Magnuson *et al*.[24] The thermocycling protocol during 22 amplification cycles was as follows: denaturation at 94°C for 1.5 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, denaturation at 94°C for 20 s, and termination with a final cycle: annealing (55°C for 1 min) and extension (72°C for 7 min). Exponential amplifications for ED-A, ED-B and IIICS were confirmed over the 22 cycles. The amplified DNA fragments were analyzed by electrophoresis on 2% agarose gels.

Immunofluorescence staining ACC3 cells at a concentration of 3×10^4 in 2 ml of medium were plated onto a 35 mm plastic dish in which a piece of cover glass was placed. Seven days after plating, the dishes were fixed and stained with an indirect immunofluorescence technique, using the anti-ED-A (IST-9) or ED-B (BC-1) segment-specific monoclonal antibodies and rhodamine-conjugated goat anti-mouse IgG (1:50, Miles Scientific, Naperville, IL), as described previously.[15] As controls, preimmune mouse IgG was used instead of the specific primary antibodies.

***In situ* hybridization** To synthesize probes for the ED-A and ED-B regions of FN, ED-A and ED-B fragments with *Eco*RI and *Bam*HI recognition sites were amplified by nested-PCR from the PCR products of ACC-FN described above. We designed primers for the ED-A and ED-B regions as follows: ED-A (forward, with *Bam*HI site), 5′-ATGGATCCGGTTCAGACTGCAGTAACCA-3′; ED-A (reverse, with *Eco*RI site), 5′-ATGAATTCCAGTTGGTG-CAGGAATAGCT-3′); ED-B (forward, with *Bam*HI site), 5′-ATGAATTCAATGTTGGTGAATCGCAGGT-3′; ED-B (reverse, with *Eco*RI site), 5′-ATGGATCCTCTCTGA-TACCATCATCCCA-3′. cDNA fragments for ED-A (324 bp) and ED-B (346 bp) were then subcloned into pSPT18, and the RNA probes were synthesized by either T7 (antisense) or SP6 (sense) RNA polymerase using digoxigenin-UTP (DIG RNA Labeling Kit (SP6/T7), Boehringer Mannheim GmbH, Mannheim, Germany).

ACC3 cells cultured on cover glasses for 7 days were fixed with 4% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.5) for 15 min. They were treated with 1 μg/ml of proteinase K (Sigma Chemical) at room temperature for 20 min, followed by washing with 0.027 *M* glycine in diethyl dicarbonate-treated PBS. The cells were then incubated with 60 μl of the hybridization solution [50% formamide, 10% dextran sulfate, 0.5× SSC, 1× Denhardt's solution, 0.1 mg/ml sonicated salmon sperm DNA and 125 μg/ml yeast tRNA] containing 500 ng/ml RNA probes in a humidified chamber for 15 h at 45°C. They were washed with 0.1 *M* maleic acid buffer (pH 7.5) containing 0.05% Triton X-100, and hybridization signals were visualized by using the DIG Nucleic Acid Detection Kit (Boehringer Mannheim). The cells on cover glasses were counterstained with methyl green and mounted on slide glasses.

Cell attachment assay Three rows of wells in a 96-well microtiter plate (Iwaki Glass Co., Ltd., Funabashi) were coated with 100 μl of serial dilutions (0.08–2 μg/ml) of ACC-FN and pFN for 30 min at 37°C, followed by blocking with 10 mg/ml bovine serum albumin (Amersham International) for 30 min at 37°C. ACC3 cells were plated at a density of 2.0×10^4 cells/well in DMEM and incubated for 30 min at 37°C. After the incubation, nonadherent cells were removed and counted. Attached cells were fixed with 4% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.4) for 20 min at room temperature, then stained with Giemsa's solution, and counted. Data are presented as mean±SD percentage of cell attachment. The statistical significance of differences in cell attachment

was analyzed by using Student's *t* test. The criterion of significance was taken as $P<0.005$ or $P<0.05$.

RESULTS

Purification of fibronectin produced by ACC3 cells A total of 1 liter of the medium from ACC3 cultures was concentrated, supplemented with proteinase inhibitors, and applied to a gelatin-Sepharose column. The column was washed, the bound material was eluted with 8 *M* urea buffer and the eluted peak was collected. The eluate was concentrated 10-fold and subjected to SDS-PAGE. The yield of FN (ACC-FN) was estimated at 2.5 mg. The purified ACC-FN gave a single band with a molecular weight of 315 kDa on SDS-PAGE under reducing conditions (Fig. 1, lane 1). This molecular weight is definitely larger than that of pFN (Fig. 1, lane 2) or any other FN reported in the literature. Under non-reducing conditions, 40% of ACC-FN molecules were dimers (630 kDa) and 60% were monomers (315 kDa) (Fig. 1, lane 3), whereas 100% of pFN was dimeric (Fig. 1, lane 4). ACC-FN seemed to be shed into the medium as monomeric forms under these conditions of culture.

Immunological studies Polyclonal antibodies against purified ACC-FN were prepared in rabbits. The anti-ACC-FN as well as the anti-pFN antibodies exhibited distinct titers of about 1:6,400 to 1:12,800 on ELISA when microtiter plate wells were coated with pFN. In order to determine more precisely the relationship between ACC-FN and pFN, we performed inhibition-ELISA assays. Dilutions of pFN were tested for their capacity to compete with the binding of either the anti-pFN or anti-ACC-FN antibodies to pFN-coated plates. Similar inhibition curves were obtained (50% inhibition at about 0.1 μg/ml of pFN) for both antibody populations (not shown) and this result indicated antigenic similarity or identity.

Immunoprecipitation analysis and immunofluorescence Since the molecular weight of ACC-FN estimated on SDS-PAGE was distinctly higher than that of pFN, we compared ACC-FN with FNs of several different cell lines obtained by immunoprecipitation with the anti-ACC-FN antibodies. ACC3, MK-1 and ZK-1 cells and fibroblasts were cultured for 6 days, and their culture media were immunoprecipitated after depletion of FCS for 1 h followed by [^{35}S]methionine labeling for 3 h in the absence of FCS. Immunoprecipitated FNs from culture media of MK-1 cells and fibroblasts showed broad bands containing doublets or triplets with molecular masses of 260−310 kDa on SDS-PAGE under reducing conditions (Fig. 2, lanes 2 and 3).

When ACC3 culture media from day 6 after seeding were immunoprecipitated with the segment-specific monoclonal antibodies IST-9 (anti-ED-A region) and BC-1 (anti-ED-B), a single band of 315 kDa was obtained with each antibody (Fig. 3A, lanes 3 and 4). This result indicated that ACC-FN contained both ED-A and ED-B regions, which should be alternatively spliced in the biosynthetic process. At the same time, ACC3 cells were immunostained with anti-ED-A or anti-ED-B monoclonal antibodies. As shown in Fig. 3B, immunofluorescence signals for the ED-A and ED-B regions of ACC-FN were localized mainly in the intercellular space. The signals

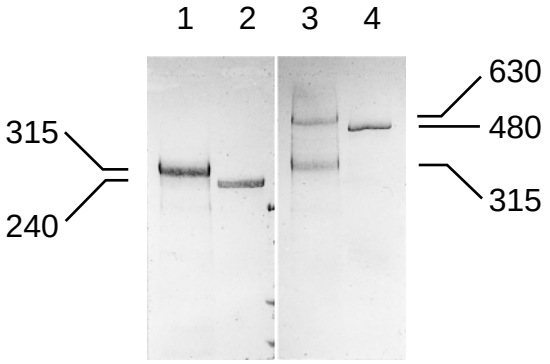

Fig. 1. SDS-PAGE of purified FN (ACC-FN) from the culture medium of ACC3 cells in comparison with that of human pFN. The gels were stained with CBB. Lane 1, ACC-FN, reduced; lane 2, pFN, reduced; lane 3, ACC-FN, not reduced; lane 4, pFN, not reduced. ACC-FN in the culture medium, consisting of monomeric (315 kDa) and dimeric (630 kDa) forms, was larger in size than pFN (240 and 480 kDa). Molecular weights (kDa) of FNs are shown on both sides.

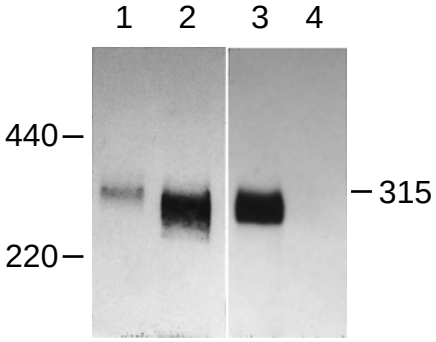

Fig. 2. Comparison of FN isoforms secreted by different cell lines. FNs were immunoprecipitated from [^{35}S]methionine-labeled culture media of ACC3 cells (lane 1), MK-1 cells (lane 2), fibroblasts (lane 3) and ZK-1 cells (lane 4) with the anti-ACC-FN. Immunoprecipitated FNs were analyzed by SDS-PAGE under reducing conditions and fluorography. Molecular mass standards in kDa are shown on the left. ACC-FN of *Mr* 315 kDa shows a sharper and larger peak than FNs from fibroblasts and MK-1 cells. There was no FN in ZK-1 cells.

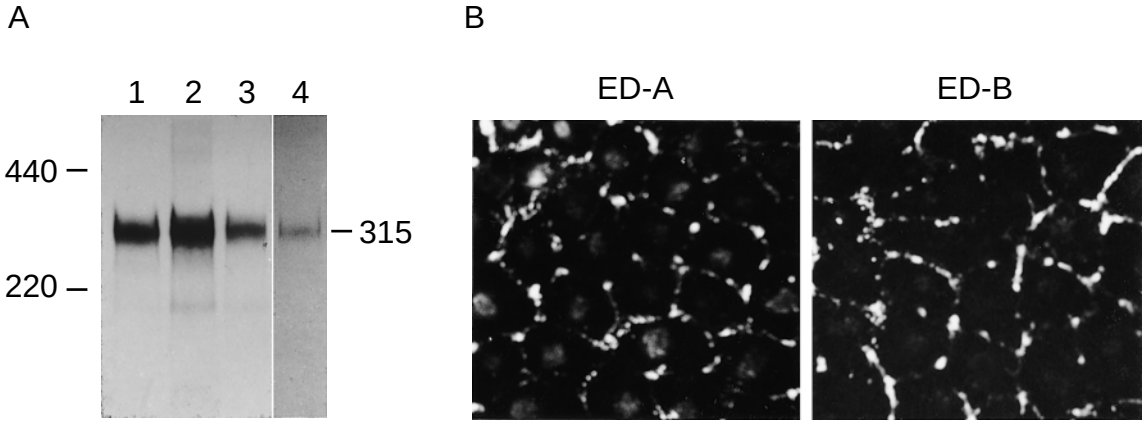

Fig. 3. Presence of both ED-A and ED-B regions in ACC-FN. Panel A. Immunoprecipitation of ACC-FN from [^{35}S]methionine-labeled ACC3 culture media with different antibodies against FN isoforms and fragments. Lane 1, anti-pFN (polyclonal); lane 2, anti-ACC-FN (polyclonal); lane 3, anti-ED-A region (monoclonal, IST-9); lane 4, anti-ED-B region (monoclonal, BC-1). Molecular masses of the standards in kDa are shown on the left. ACC-FN of *Mr* 315 kDa shares its antigenic sites with pFN, and contains both ED-A and ED-B regions. Panel B. Immunofluorescence for ED-A (left) and ED-B (right) regions of FN in ACC3 cells at day 6 after seeding.

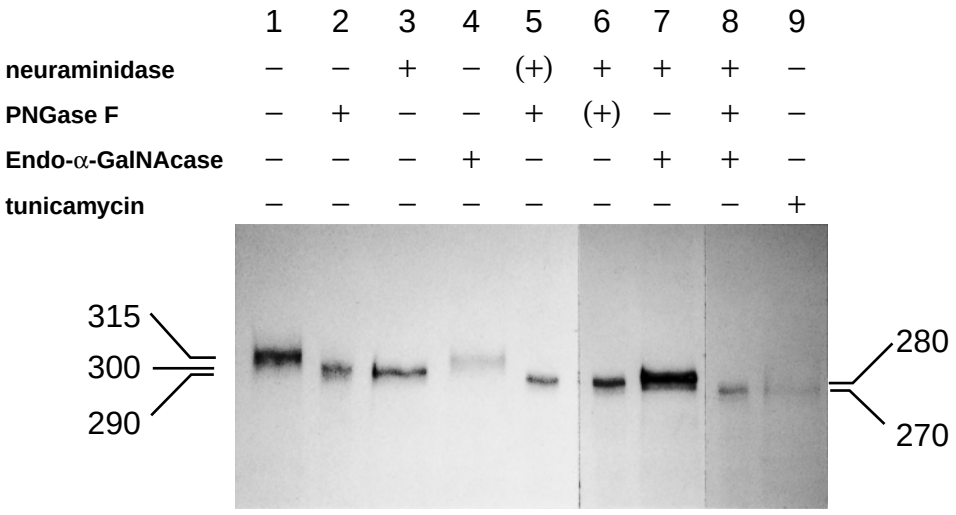

Fig. 4. Enzymatic digestion of oligosaccharide chains of ACC-FN. ACC-FN was immunoprecipitated from [^{35}S]methionine-labeled ACC3-conditioned media and analyzed by SDS-PAGE and fluorography. ACC-FN was sequentially digested with combinations of neuraminidase, PNGase F and endo-α-N-acetylgalactosaminidase as indicated by +/(+)/− (precedent treatment in parentheses). Reduction of the molecular mass of ACC-FN was differentially obtained by treatments with PNGase F (lane 2, 300 kDa), neuraminidase (lane 3, 290 kDa), endo-α-N-acetylgalactosaminidase (lane 4, 315 kDa), neuraminidase and then PNGase (lane 5, 280 kDa), PNGase F and then neuraminidase (lane 6, 280 kDa), neuraminidase and then endo-α-N-acetylgalactosaminidase (lane 7, 280 kDa) and neuraminidase, endo-α-N-acetylgalactosaminidase, and then PNGase F (lane 8, 270 kDa). The molecular mass of ACC-FN was reduced to 270 kDa by tunicamycin treatment (lane 9).

were regarded as reflecting extracellular deposition of ACC-FN secreted by ACC3 cells.

Enzymatic digestion of oligosaccharide chains of ACC-FN In addition to the alternative splicing, posttranslational modifications of ACC-FN were considered to be another possible explanation for the higher molecular weight of ACC-FN. We therefore analyzed the glycosylation patterns of this molecule by the use of several exo-

and/or endo-glycosidases. Digestion of ACC-FN with PNGase F, which cleaved off N-glycans, resulted in a marked reduction of the molecular mass of ACC-FN, yielding a rather broad band migrating at around *Mr* 300 kDa (Fig. 4, lane 2). Treatment with neuraminidase alone resulted in a marked reduction of the molecular mass, yielding a sharp band of 290 kDa (Fig. 4, lane 3). When ACC-FN was treated with endo-α-N-acetylgalactosaminidase, ACC-FN was definitely not reduced in molecular weight (Fig. 4, lane 4). However, when ACC-FN was first desialylated with neuraminidase, endo-α-N-acetylgalactosaminidase successfully removed desialylated O-linked glycans, producing a smaller form of 280 kDa (Fig. 4, lane 7). Sequential treatment of the 280-kDa form with PNGase F resulted in a further reduction in size to 270 kDa (Fig. 4, lane 8). When desialylated ACC-FN was treated with PNGase F, the molecular weight of ACC-FN was reduced to 280 kDa (Fig. 4, lane 5). Digestion of PNGase F-treated ACC-FN with neuraminidase yielded a smaller and less heterogeneous band of 280 kDa (Fig. 4, lane 6). Since the N-glycans of ACC-FN had already been removed by PNGase F, the results suggest that the O-linked oligosaccharide chains on ACC-FN were sialylated. When ACC3 cells were treated with tunicamycin, ACC-FN migrated to the same position as the deglycosylated form (Fig. 4, lane 9). These results indicated that the ACC-FN polypeptide has a molecular mass of 270 kDa, and that ACC-FN contained about 10 kDa of sialylated O-linked oligosaccharides, as well as about 10 kDa of N-linked oligosaccharides.

Kinetics of glycosylation of ACC-FN The processes of addition of oligosaccharide chains were examined by means of a pulse/chase experiment. ACC3 cells were pulse-labeled for 30 min with [^{35}S]methionine and chased for 30 to 180 min with unlabeled methionine. At the end of chase, cell lysates and media were immunoprecipitated and analyzed on SDS-PAGE under reducing conditions. After pulse-labeling for 30 min, a major band of 290 kDa was detected in the cell lysate. This was considered as an immature form of ACC-FN, corresponding to a polypeptide containing both N-linked and O-linked oligosaccharide chains without sialic acid (Fig. 5, lane 1). At 30 min of chase, a 315-kDa high-molecular-weight form emerged, in addition to the 290-kDa form (Fig. 5, lane 2). At 60 min of chase, the 315-kDa band became more distinct, while the 290-kDa band was diminished (Fig. 5, lane 3). At 120 min, only the 315-kDa band was detectable (Fig. 5, lane 4), and it became faint at 180 min in the cell lysate (Fig. 5, lane 5). On the other hand, in the medium, a small amount of the 315-kDa form appeared first at 120 min of chase (Fig. 5, lane 9), and it increased

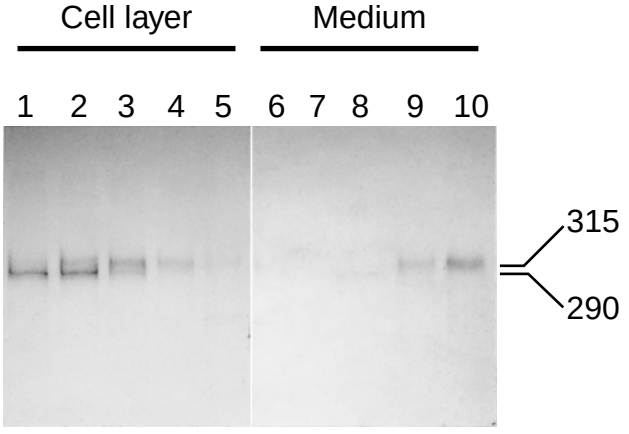

Fig. 5. Maturation process of oligosaccharide chains of ACC-FN. ACC3 cells were labeled for 30 min with [^{35}S]methionine (lanes 1 and 6) and then chased for 30 min (lanes 2 and 7), 60 min (lanes 3 and 8), 120 min (lanes 4 and 9) and 180 min (lanes 5 and 10) in the medium containing unlabeled methionine. Radiolabeled ACC-FN was immunoprecipitated from cell lysates (lanes 1–5) and culture media (lanes 6–10) and analyzed by SDS-PAGE and fluorography. The maturation process of ACC-FN, from immature (lanes 1–3, 290 kDa) to mature forms (lanes 2–5, 315 kDa) can be clearly seen in a time-dependent manner. Only mature forms are secreted into the media (lanes 9, 10).

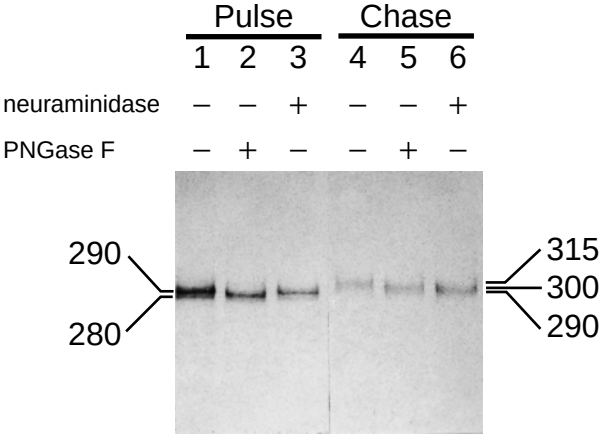

Fig. 6. Sialylation in the maturation process of N-linked oligosaccharide chains of ACC-FN. ACC3 cells were pulse-labeled with [^{35}S]methionine for 30 min and then chased with non-labeled methionine for 60 min. Radiolabeled ACC-FN was immunoprecipitated from cell lysates, then treated with neuraminidase or PNGase F as indicated at the top, and analyzed by SDS-PAGE and fluorography. Pulse-labeled ACC-FN (290 kDa, lane 1) is reduced in size by treatment with PNGase F (280 kDa, lane 2), but is not sensitive to neuraminidase (290 kDa, lane 3). However, chased ACC-FN was sensitive to both PNGase F (300 kDa, lane 5) and neuraminidase (290 kDa, lane 6).

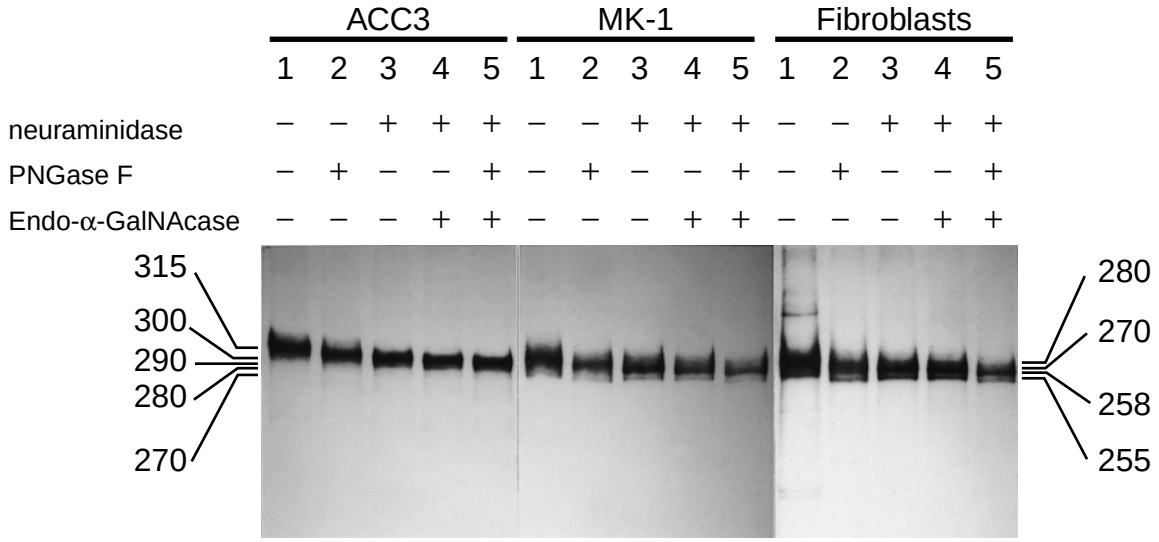

Fig. 7. Comparison of oligosaccharide chains on FNs from different cell lines. FNs secreted by ACC3, MK-1 and fibroblasts were immunoprecipitated with the anti-ACC-FN from [^{35}S]methionine-labeled culture media. FNs were sequentially digested with PNGase F (lane 2), neuraminidase (lane 3), combinations of neuraminidase and endo-α-N-acetylgalactosaminidase (lane 4) or further with PNGase F (lane 5). Molecular sizes were reduced by treatments with PNGase F (ACC3, 300 kDa; MK-1, 270–260 kDa; fibroblasts, 260–255 kDa), neuraminidase (ACC3, 290 kDa; MK-1, 260–255 kDa; fibroblasts, 260–255 kDa), neuraminidase+endo-α-N-acetylgalactosaminidase (ACC3, 280 kDa; MK-1 and fibroblasts, 260–255 kDa) and neuraminidase+endo-α-N-acetylgalactosaminidase+PNGase F (ACC3, 270 kDa; MK-1 and fibroblasts, 258–255 kDa).

significantly at 180 min. No other smaller bands were observed in the medium (Fig. 5, lane 10).

To determine N-glycosylation and sialylation of ACC-FN, ACC3 cells were labeled with [^{35}S]methionine for 30 min, and chased for 60 min. Immunoprecipitated cell lysates were digested with PNGase F or neuraminidase. Pulse-labeled ACC-FN digested with PNGase F migrated faster (280 kDa) than that without digestion (290 kDa) (Fig. 6, lanes 1 and 2). However, digestion with neuraminidase did not affect the migration of ACC-FN on SDS-PAGE (Fig. 6, lane 3). On the other hand, ACC-FN chased for 90 min was sensitive to both PNGase F and neuraminidase and migrated faster than that without digestion (Fig. 6, lanes 4–6). These data indicated that N-glycosylated ACC-FN was not yet sialylated at 30 min, but that modification of the oligosaccharide chains of ACC-FN with sialic acid was completed by 90 min, and this would support the interpretation of Fig. 5 described above.

Comparison of oligosaccharide chains of FNs from different cell lines Since there were differences in the size of FNs between ACC3 cells, MK-1 cells and fibroblasts (Fig. 2), we compared the mode of glycosylation of FNs from the other cell lines by means of enzymatic digestions as described above. As in the case of ACC3, the molecular mass of PNGase F-treated FNs of MK-1 cells (270–260 kDa) was larger than that after neuraminidase treatment (260–255 kDa) (Fig. 7, MK-1: lanes 2 and 3). However, in fibroblasts, PNGase F-treated FNs were almost equal in size to those treated with neuraminidase (260–255 kDa) (Fig. 7, fibroblasts: lanes 2 and 3). Sequential treatments of FNs from MK-1 and fibroblasts with the three enzymes resulted in a marked reduction in molecular mass, yielding less heterogeneous doublet bands migrating at *Mr* 258 and 255 kDa (Fig. 7, MK-1: lane 5, fibroblasts: lane 5), respectively. These results indicate that the deglycosylated polypeptide chain of ACC-FN is larger and less heterogeneous than that of FNs of MK-1 and fibroblasts.

Alternative splicing of FNs Since the sizes of deglycosylated FNs were different between ACC3, MK-1, and fibroblasts as described above, we examined the modes of alternative splicing of FN-mRNA in the four cell lines, ACC3, MK-1, fibroblasts, and ZK-1. Total RNAs were extracted from each cell culture and 3 μg of each of them was reverse-transcribed with oligo-dT primers. These cDNAs were amplified with two oligonucleotide primer pairs, eda-1/eda-2 and edb-1/edb-2, designed from the exons flanking the ED-A or ED-B regions, respectively (Fig. 8A), to detect the spliced-in (ED-A(+), 604 bp; ED-B(+), 775 bp) and the spliced-out (ED-A(–), 334 bp; ED-B(–), 502 bp) FN m-RNA isoforms. In ACC3 cells, >99%

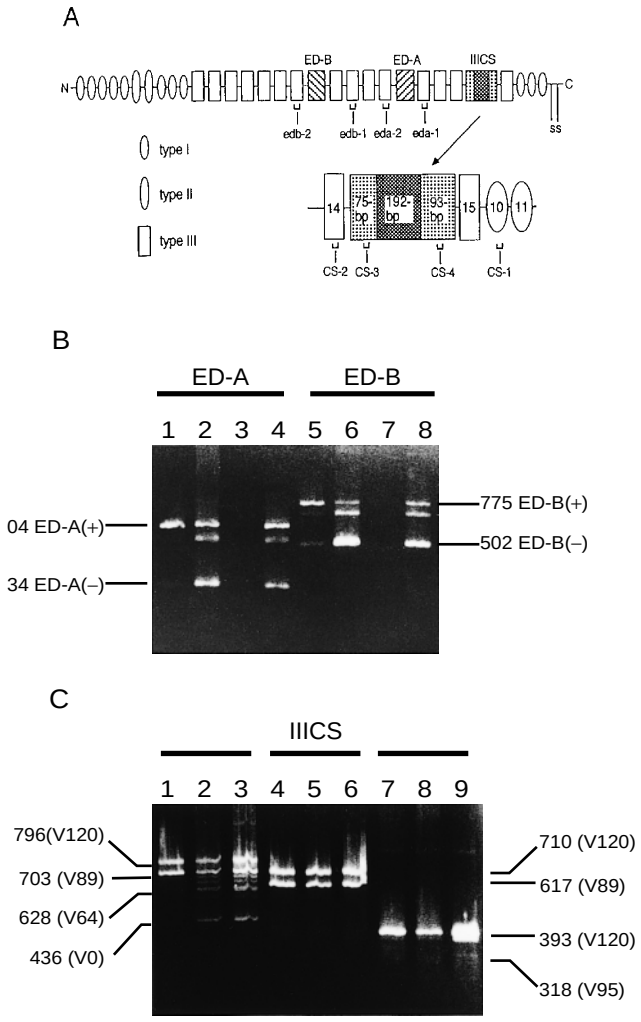

Fig. 8. Alternative splicing for ED-A, ED-B and IIICS regions of ACC-FN. Panel A. Molecular schema of FN primary structure indicating the positions of exons ED-A, ED-B, and IIICS, which are spliced in or out during RNA processing. Primers were designed for the regions indicated by brackets. Panel B. RT-PCR products for the ED-A and ED-B regions from ACC3 (lanes 1 and 5), MK-1 (lanes 2 and 6), ZK-1 (lanes 3 and 7) and fibroblasts (lanes 4 and 8) electrophoresed in 2% agarose gels. PCR products at 604, 334, 775 and 502 bp correspond to ED-A(+), ED-A(–), ED-B(+) and ED-B(–) mRNA isoforms, respectively. Panel C. RT-PCR products for the IIICS region of ACC3 (lanes 1, 4 and 7), MK-1 (lanes 2, 5 and 8) and fibroblasts (lanes 3, 6 and 9) were analyzed. Five types of alternative splicing of the three extra segments (75, 192 and 93 bp) were confirmed by RT-PCR using three combinations of four primers. When CS-1 and CS-2 were used, PCR products of 796, 703, 628 and 436 bp corresponded to the V120, V89, V64 and V0 IIICS isoforms, respectively (Fig. 8C, lanes 1–3). When CS-1 and CS-3 were used, PCR products of 710 and 617 bp corresponded to V120 and V89 isoforms (Fig. 8C, lanes 4–6). When CS-2 and CS-4 were used, PCR products of 393 and 318 bp corresponded to V120 and V95 isoforms (Fig. 8C, lanes 7–9).

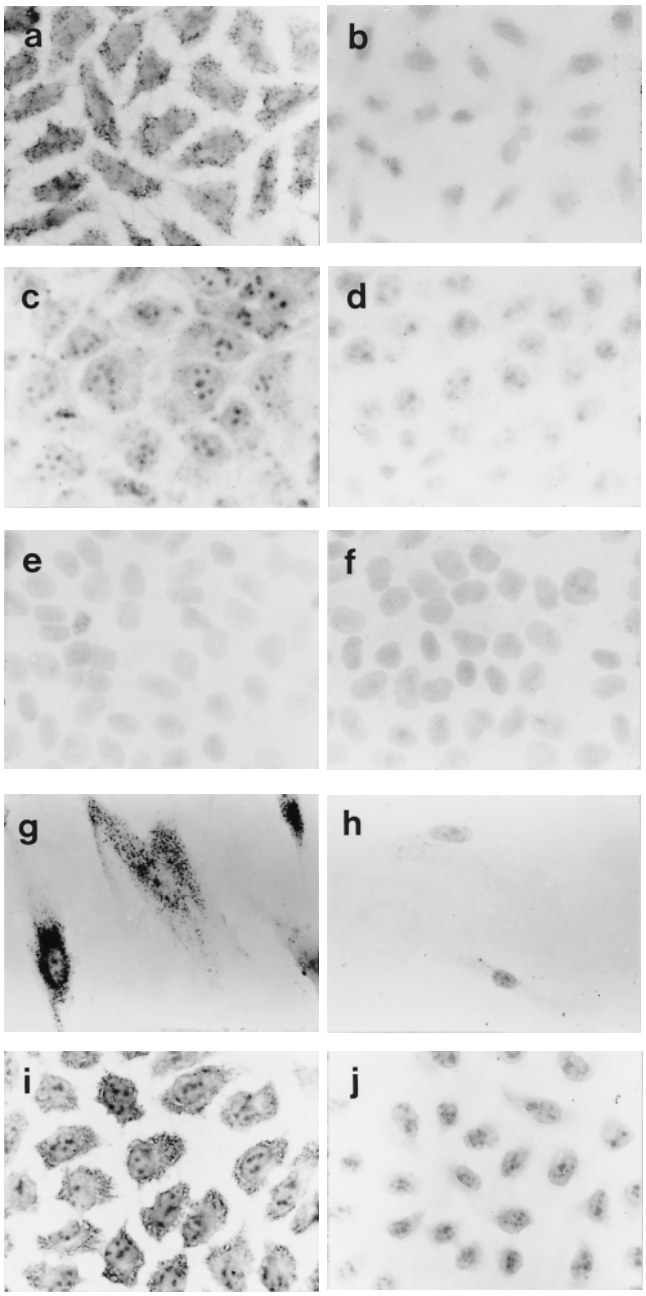

Fig. 9. *In situ* hybridization for FN-mRNA containing ED-A and ED-B regions. ACC3 (a, b, i, j), MK-1 (c, d), ZK-1 (e, f) and fibroblasts (g, h) were cultured for 5 days and hybridized with anti-sense (right column) or sense (left column) RNA probes for ED-A (a–h) or ED-B (i, j) regions of FN isoforms. Hybridized products were visualized with the digoxigenin-immunoalkaline-phosphatase method. Positive cytoplasmic signals for the ED-A region were detected in most of the ACC3 cells (a), but in fewer of the MK-1 cells (c) and fibroblasts (g). No significant signals were obtained in ZK-1 cells (e). Those for the ED-B region were seen in most of the ACC3 cells (i).

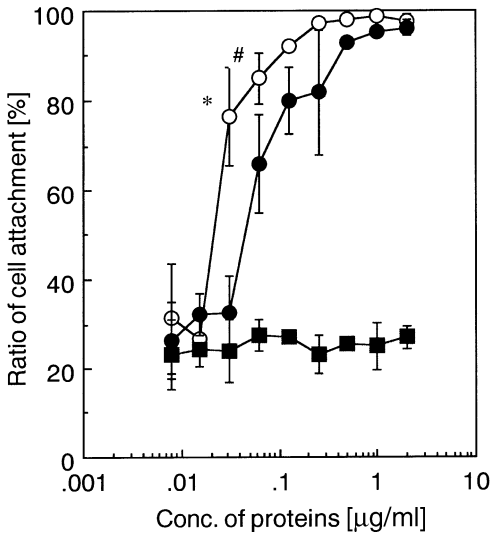

Fig. 10. Attachment assay of ACC3 cells on ACC-FN and pFN. ACC3 cells were seeded at a density of 2×10^4 cells/well on microtiter plates coated with serial dilutions of ACC-FN (open circles), pFN (closed circles) and BSA (open squares) and incubated for 30 min at 37°C. After incubation, attached cells were fixed, stained and counted. At the same time, non-adherent cells were counted. Ratios of attached cells were calculated in triplicate. Bar, mean±SD. Significant differences ($*P<0.005$, $\#P<0.05$) from the value for pFN were found by Student's *t* test.

of FN mRNA isoforms were ED-A(+) (604 bp) and >95% of them were ED-B(+) (775 bp) (Fig. 8B, lanes 1 and 5). However, in MK-1 cells, equivalent amounts of ED-A(+) (604 bp) and ED-A(−) (334 bp) FN mRNA isoforms were observed (Fig. 8B, lane 2). Approximately 70% of FN mRNA isoforms obtained from fibroblasts were ED-A(+) and 30% were ED-A(−) (Fig. 8B, lane 4). In MK-1 cells as well as in fibroblasts, ED-B(+) mRNAs (775 bp) were small but 90% of them were ED-B(−) (502 bp) (Fig. 8B, lanes 6 and 8). No PCR products for ED-A and ED-B segments were detected in ZK-1 cells (Fig. 8A, lanes 3 and 7). Similar non-specific amplification products were observed in PCR for the ED-A and ED-B regions in MK-1 cells and fibroblasts (Fig. 8B, lanes 2, 4, 6 and 8).

In order to examine alternative splicing modes of the IIICS region of FN mRNA in the three cell lines other than ZK-1, we used three combinations of four PCR primers as follows: CS-1, located in exon I-10, CS-2 in exon III-14, CS-3 in the 75-bp extra segment within the IIICS region, and CS-4 in the 93-bp extra segment (Fig. 8A). When CS-1 and CS-2 were used, equal amounts of 796-bp (referred to as V120, composed of 192-, 93- and 75-bp extra segments) and 703-bp (V89, composed of 75- and 192-bp) isoforms were found in ACC3 cells (Fig. 8C, lane 1). In MK-1 cells and fibroblasts, small amounts of 436 bp (V0, IIICS spliced-out) and 628 bp (V64, composed of 192-bp) were detected, in addition to V120 and V89 (Fig. 8C, lanes 2 and 3). When CS-1 and CS-3 were used to detect V120 and V89 isoforms, equal amounts of 710 bp (V120) and 617 bp (V89) were revealed in all three of the cell lines (Fig. 8C, lanes 4–6). When CS-2 and CS-4 were used to detect V120 and V95 isoforms, 393 bp (V120) was found in all of them (Fig. 8C, lanes 7–9). However, a small amount of 318 bp (V95) was additionally found in fibroblasts (Fig. 8C, lane 9).

These results indicate that the constitution of mRNA isoforms of ACC-FN was less heterogeneous than that of FNs of MK-1 and fibroblasts.

***In situ* hybridization for ED-A/ED-B mRNA** Since differential expression of ED-A(+)/ED-B(+) FN mRNAs had been demonstrated in the four kinds of cells by RT-PCR analysis, we sought to confirm the transcription of these two FN mRNA isoforms in individual cells by *in situ* hybridization using digoxigenin-labeled RNA probes for the ED-A and ED-B regions. ACC3, MK-1, ZK-1 cells and fibroblasts cultured on poly-L-lysine-coated cover glasses for 5 days were fixed and hybridized with antisense or sense RNA probes. Most ACC3 cells showed diffuse or fine granular signals for ED-A/ED-B in the cytoplasm (Fig. 9, a and i), while positive signals were obtained in less than half of MK-1 cells and fibroblasts (Fig. 9, c and g). No obvious signals were found in ZK-1 cells (Fig. 9e). When sense probes were used, there were no detectable signals in the cells (Fig. 9, b, d, f, h, and j).

Cell adhesive activity of ACC-FN To explore the physiological functions of the high-molecular-weight form of FN synthesized by ACC3 cells, we compared cell-adhesive activity between ACC-FN and pFN. When ACC3 cells were seeded in microtiter plates coated with ACC-FN or pFN, there was a significant difference in the number of cells attached by 30 min between the two types of FNs, in a dose-dependent manner. On ACC-FN-coated plates, 75% of cells were attached at a concentration of 0.03 μg/ml and 100% at 0.25 μg/ml, whereas a concentration of more than 0.5 μg/ml of pFN was needed for 100% attachment. ACC-FN was more potent than pFN in promoting attachment of ACC3 cells in the concentration range of 0.016–0.25 μg/ml (Fig. 10). The difference between the two coating conditions was statistically significant in the range of 0.031 ($P<0.005$) to 0.061 ($P<0.05$) μg/ml. The result indicated that an FN molecule with ED-A/ED-B/IIICS regions and larger amounts of N- and O-linked oligosaccharide chains functions as a substrate to which ACC3 cells can readily become attached.

DISCUSSION

The present study shows that ACC3 cells, established from a human adenoid cystic carcinoma, produce a high-

molecular-weight isoform (315 kDa) of FN (ACC-FN). This large form partly results from alternative splicing with the ED-A, ED-B, and IIICS segments and partly from glycosylation with both O-linked and N-linked oligosaccharide chains. Furthermore, this special isoform of FN may contribute to the attachment of ACC3 cells. As we have shown in previous studies, ACC3 cells are capable of producing FN and at least four basement membrane constituents, type IV collagen, laminin, entactin, and heparan sulfate proteoglycan.[14, 15] Among these molecules, FN is most plentifully synthesized by ACC3.[14] Thus, the primary aim of this study was to characterize the biochemical properties of FN produced by ACC3 cells.

The larger molecular weight, 315 kDa, of ACC-FN was shown to arise partly from post-translational modification with sugar chains. Since 10 kDa each of N-linked and O-linked oligosaccharide chains are attached to the polypeptide of 270 kDa, ACC-FN should have a molecular mass of at least 290 kDa. The value of 315 kDa for the molecular mass of ACC-FN may not be accurate, because the high negative charge due to sialic acid on the oligosaccharide chains interferes with the accurate estimation of sizes of glycoproteins on SDS-PAGE. The amount of 10 kDa of N-linked oligosaccharide chains on ACC-FN is similar to that of human pFN.[25] However, that of human placental FN is two times larger (20 kDa), because these chains are composed of tri- and heteroantennary, in addition to biantennary chains.[11] The present data indicate that the N-linked oligosaccharide chains of ACC-FN have little or no sialylation. In contrast, most of the N-linked sugar chains are sialylated in pFN and approximately half of them are sialylated in placental FN,[26] although the significance of sialylation of N-linked oligosaccharide chains is poorly understood. On the other hand, there are no data on the amounts of O-linked oligosaccharide chains attached in these FN isoforms. However, Matsuura *et al.* demonstrated by using a monoclonal antibody that sialylated O-linked oligosaccharide chains were located in the IIICS region of FNs isolated from fetal connective tissues and various malignant cells and tissues, and that they were not present in pFN and FNs from normal adult tissues.[27, 28] Therefore, it is possible to regard ACC-FN as an oncofetal molecule, although we did not determine in detail the structures and attachment sites of the oligosaccharide chains of ACC-FN.

When compared with the FN polypeptides of MK-1 cells and fibroblasts, that of ACC-FN was larger and less heterogeneous. Several lines of evidence have indicated that the molecular diversity of FNs is mainly derived from the tissue-specific alternative splicing of their mRNAs, although *in-vivo* FN mRNA regulation has been poorly documented.[6, 7] Using the semi-quantitative RT-PCR method, we have shown that the difference in molecular mass of FNs between the three cell lines results partly from alternative splicings at the ED-A, ED-B and IIICS regions. Higher frequencies of ED-A and ED-B regions were noticeable in the total FN mRNA from ACC3 cells. The expression of the ED-A and ED-B regions in ACC3 cells was also confirmed by immunofluorescence and *in-situ* hybridization studies. If human cellular FN mRNA contained the ED-A and ED-B regions and 360 bp or 267 bp of IIICS segments, the gene product would be estimated to be 272 or 269 kDa in molecular mass. This molecular weight is close to that of ACC-FN observed in the present immunoprecipitation experiments. Enhanced expressions of ED-A(+) and/or ED-B(+) FN isoforms have been reported in human fetal liver tissues and liver cell carcinomas[29] and in rat wound healing processes[3] or hypertrophic cardiac tissues.[30] Since the expression of ED-A(+) FN isoforms is basically low in normal adult tissue, enhanced expression could be considered to be an oncofetal sign. In contrast to the ED-A segment that is constitutively expressed to some extent in adult and fetal tissues, a profound difference has been observed in the level of the ED-B(+) mRNA between normal and neoplastic tissues.[31] The exclusive expression of the ED-B region in ACC-FN suggests a neoplastic nature of the FN isoform.

Although the functions of the ED-A and ED-B regions have not been well elucidated, these segments might play important roles in the extracellular milieu because they are highly conserved among species. It was reported that FNs containing ED-A with/without ED-B segments were approximately twice as potent as those lacking ED-A in their abilities to promote cell adhesion and migration.[32] It has also been observed that ED-A-enriched cellular FN promotes adhesion of human synovial cells more than pFN lacking ED-A.[33] Our present result, showing that ACC-FN is more potent than pFN for adhesion of ACC3 cells, is consistent with these data. On the other hand, the immunohistochemical findings suggested that ED-B(+) FNs are associated with angiogenesis. The angiogenic potency of ED-B(+) FNs is likely to explain the frequent metastasis on hematogenous routes of adenoid cystic carcinomas.

The pulse/chase experiments showed that the time courses of glycosylation and secretion of ACC-FN were significantly delayed when compared with those of FNs from other cell types reported previously. Choi and Hynes reported that newly synthesized FN molecules began to appear in the culture medium 30 min after labeling of hamster fibroblastic cells.[34] Villigar *et al.* obtained a similar result in human alveolar macrophages.[35] Based on the present time course data, the process of sialylation of O-linked oligosaccharide chains seems to account for the retarded secretion of ACC-FN. In the previous immunofluorescence study using a confocal microscope, FN had been found to be localized prominently in the pericellular

space of ACC3 cell layers.[15] It is hence suggested that newly synthesized ACC-FN molecules are accumulated in the cell layer, but are not secreted immediately into the culture medium from ACC3 cells. Such an ability of ACC3 cells to trap FN molecules on the cell surface may be another reason for the retarded secretion of ACC-FN. This characteristic should also be important in forming the characteristic histologic architecture of adenoid cystic carcinoma.

ACKNOWLEDGMENTS

The authors are grateful to Dr. K. Sekiguchi, Osaka University Institute for Protein Research, for his generous supply of the antibodies. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan.

(Received October 26, 1998/Revised December 11, 1998/ Accepted December 16, 1998)

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