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The oncofetal domain of fibronectin defined by monoclonal antibody FDC-6: Its presence in fibronectins from fetal and tumor tissues and its absence in those from normal adult tissues and plasma

(hepatoma/COOH-terminal region)

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ABSTRACT **An IgG1 monoclonal antibody, FDC-6, was established, which defines a unique fibronectin (FN) domain, located between the "Hep-2" and the "Fib-2" domains, in the COOH-terminal region of FNs isolated from hepatoma, sarcoma, and fetal fibroblasts. A systematic study with this antibody indicates the presence of two classes of human FNs. (*i*) FN from fetal connective tissue, placenta, amniotic fluid, hepatoma, and colon carcinoma as well as cell lines from fetal tissues (WI-38), hepatomas (HuH-6 and HuH-7), and sarcoma (VA13) was characterized by the presence of the FDC-6-defined domain and by a high molecular weight (subunit M_r, 310,000–335,000). (*ii*) In contrast, FN from normal adult tissues and plasma was characterized by a lower molecular weight (subunit M_r, 285,000–295,000) and lack of reactivity with FDC-6 and is therefore devoid of the FDC-6-defined domain. The FDC-6-defined domain is therefore called the "oncofetal" domain, and FN containing this domain is hereby called "*oncofetal FN*" (onf FN). The onf FN is similar to the previously known "cellular-form" FN. FN from normal adult tissues and plasma, lacking the oncofetal domain, is hereby called "*normal FN*" (nor FN). The nor FN is similar to the previously known "plasma-form" FN. Development of FN from fetal to adult form is associated with loss of the oncofetal domain defined by the FDC-6 antibody, and oncogenic transformation is associated with activation in synthesis of the oncofetal domain defined by the FDC-6 antibody.**

Since fibronectin (FN) is the major component of the pericellular matrix, is greatly reduced on oncogenic transformation (1–3), and displays multifunctional properties, including opsonic activity in plasma, extensive and diversified studies have been developed toward understanding the role of this macromolecule in the structure and function of the pericellular matrix as well as in homeostasis of "milieu intérieur" (for reviews, see refs. 4–11). One line of study undertaken by us (12–17) and others (18–22) has been to compare the domain structures of FNs from fibroblasts with those from plasma. FNs from these two sources show clear differences in molecular weight (size of subunits), particularly in the COOH-terminal region defined by monoclonal antibody IST-1 (15–17). FNs from fibroblasts, fibrosarcoma, and hepatoma are consistently larger by a M_r of 25,000–40,000 in the COOH-terminal domain than FN from plasma (plasma-form FN) (16, 17). A monoclonal antibody, FDC-6, has been established, the hybridoma of which was selected by positive reactivity with FNs from hepatoma, fibrosarcoma, and fetal fibroblasts (WI-38) and negative reactivity with FN from plasma. The antibody defines a specific domain that is present in FNs from tumors and fetal tissues but is absent in those from normal adult tissues and plasma. A preliminary characterization of this domain is hereby reported.

MATERIALS AND METHODS

Monoclonal antibody FDC-6 was established after immunization of BALB/c mice with FN isolated from HuH-6 hepatoma cells, and the hybridoma was selected by positive reactivity with FNs from human hepatoma HuH-6 and HuH-7 (17) and WI-38 fibroblasts and by negative reactivity with plasma-form FN. HuH-6 and HuH-7 cells were donated by Jiro Sato (Okayama University, Okayama, Japan). The location of the reactive site of the FDC-6 antibody was determined by limited proteolysis of FNs from plasma, WI-38 fibroblasts, and HuH-6 and HuH-7 hepatomas by thermolysin, trypsin, and cathepsin D, followed by electrophoretic transfer blotting with various antibodies directed to Fib-1/Hep-1, Gel, Hep-3/Cell, Hep-2, and Fib-2 domains (15, 16). Solid-phase radioimmunoassay with antibody and antigen dilution as well as competitive inhibition with antigen was performed as described (23). FNs from plasma and from the culture medium of fibroblasts, hepatoma, and fibrosarcoma cells were prepared by gelatin-Sepharose column chromatography as described (24). Tissue FN fractions were prepared by their insolubility in neutral detergent under nonreducing conditions—i.e., tissues were minced with scissors and homogenized in a Dounce homogenizer in phosphate-buffered saline (pH 7.2) containing 0.5% Nonidet P-40 detergent, 0.1% $NaDodSO_4$, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM EDTA. The homogenate was centrifuged, and the precipitate was washed twice in phosphate-buffered saline. The washing was followed by centrifugation at 10,000 × *g* for 30 min. The precipitate thus obtained was suspended and dissolved by heating in a boiling water bath for 5 min in "sample buffer" containing 5% 2-mercaptoethanol and 2% $NaDodSO_4$ (25). The tissue extract containing FN thus prepared was analyzed on gel electrophoresis in 0.2% $NaDodSO_4$ employing 9.5% and 6.5% polyacrylamide under reducing conditions, followed by transfer onto nitrocellulose sheets and blotting with monoclonal antibodies (16, 17, 26). The antibodies IST-1 (27), which defines the "Hep-2" domain (15, 16), and IST-4 (27), which defines a part of the "cell" domain, were donated by Luciano Zardi and Annalisa Siri (Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy). Another IgG1 antibody, FDZ, was established, which had a similar reactivity to that of IST-4 (27) and defines a part of the "cell" domain. Since the binding activity of the FDZ

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Abbreviations: FN, fibronectin; onf FN, oncofetal FN; nor FN, normal FN.

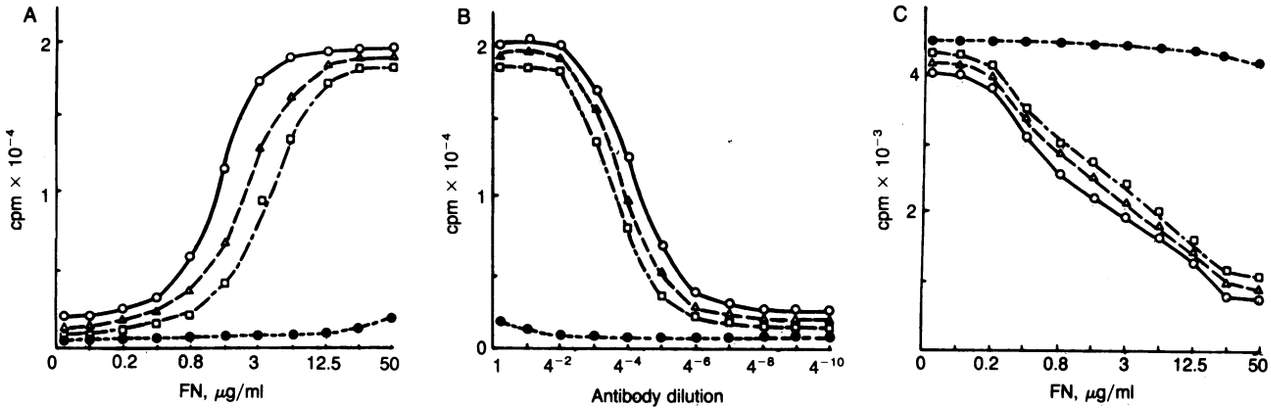

FIG. 1. Binding specificity of monoclonal antibody FDC-6. (*A*) Binding activity of the antibody with different concentrations of FNs adsorbed on a plastic surface. ●---●, Plasma-form FN; ○—○, FN isolated from culture medium of hepatoma HuH-7; △---△, FN from culture medium of hepatoma HuH-6; □- - -□, FN from culture medium of WI-38 fibroblasts. (*B*) Antibody binding activity with various concentrations of antibody to various FNs coated with a constant concentration (50 μg/ml). Symbols are the same as in *A*. (*C*) Competitive inhibition of antibody binding to hepatoma FN (HuH-7) coated on a plastic surface with a concentration of 50 μg/ml. The culture supernatant of FDC-6 cells diluted 1000 times was used as antibody. Aliquots (50 μl) of diluted antibody were mixed with increasing concentrations of various FNs followed by transfer of the mixture onto a well coated with FN from HuH-7 hepatoma. Symbols are the same as in *A*.

antibody with FNs from various sources correlates well with the chemical quantity blotted onto nitrocellulose sheets, the chemical quantity of each FN was determined on gel electrophoresis followed by electrophoretic transfer blotting (26). Multiple samples separated on gels and blotted onto nitrocellulose sheets reacted with the FDC-6 antibody as well as the FDZ antibody. Simultaneously, 1–5 μg of purified FN from plasma and HuH-7 hepatoma (17) was blotted onto nitrocellulose sheets after $NaDodSO_4$ gel electrophoresis. Thus, the binding activity of FDZ and FDC-6 can be correlated with the chemical quantity of FN. The binding activity with omission of FN and that with omission of the primary antibody were used as the background to be subtracted. To determine whether the FDC-6 antibody reacts with a carbohydrate determinant, FN coated on a plastic surface was treated with sodium periodate/borohydride (28) followed by reaction with the FDC-6 antibody as described above.

RESULTS

Specificity and Domain Recognition of Monoclonal Antibody FDC-6. The murine IgG1 monoclonal antibody FDC-6 showed reactivity with FNs from human HuH-6 and HuH-7 hepatomas and WI-38 fibroblasts. However, it did not react with human plasma FN in the assay systems with antigen dilution (Fig. 1*A*), antibody dilution (Fig. 1*B*), or inhibition of antibody binding on solid-phase FN (Fig. 1*C*).

The fragments defined by FDC-6 released by trypsin and cathepsin D were similar to but obviously different from those defined by the IST-1 antibody (16), since none of the fragments released by thermolysin was reactive with the FDC-6 antibody (Fig. 2*B*, Thermolysin). Neither the Hep-2 domain (M_r 30,000–40,000 reactive with antibody IST-1) nor the Fib-2 domain (M_r 22,000) released by thermolysin nor the Fib-2 domain (M_r 37,000) released by trypsin treatment was reactive with FDC-6, whereas the COOH-terminal region released by cathepsin D (M_r 85,000 and M_r 110,000), which showed disulfide-dependent linkage (16), was reactive with FDC-6 (Fig. 2*B*, Cathepsin D). A large M_r 200,000 fragment and M_r 120,000–160,000 and M_r 55,000–65,000 degradation products released by trypsin treatment were reactive with FDC-6 (see Fig. 2*B*, Trypsin). These FDC-6-positive trypsin fragments represent the COOH-terminal side of the middle domain after cleavage of the M_r 37,000 fragment (possible cleavage points indicated by arrows in Fig. 5*B*). Thus, the region reactive with the FDC-6 antibody must be located between the Hep-2 and Fib-2 domains within the COOH-terminal region of FNs isolated from the culture medium of hepatoma and WI-38 fibroblasts (see *Discussion*). This domain with FDC-6 reactivity is highly susceptible to digestion with thermolysin and is not detectable after thermolysin digestion. The FDC-6 reactivity is resistant to treatment with sodium periodate/borohydride and may represent a polypeptide region (see *Discussion*).

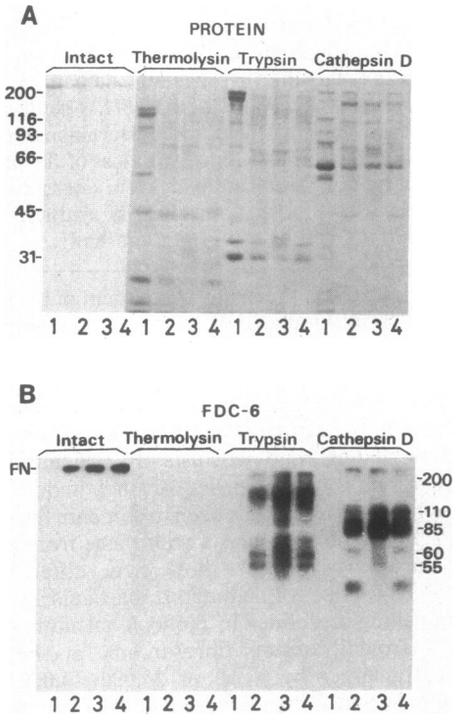

FIG. 2. Immunoblotting pattern of proteolytic fragments of various FNs with monoclonal antibody FDC-6. (*A*) Protein staining with fast green. (*B*) Immunoblotting pattern of the same gel as in *A* with monoclonal antibody FDC-6. Lanes 1, plasma FN; lanes 2, FN from culture medium of WI-38 fibroblasts; lanes 3 and lanes 4, FN from culture media of hepatoma HuH-6 and HuH-7, respectively. The fragmentation pattern induced by each enzyme is identified at the top. Molecular weights are shown as $M_r \times 10^{-3}$.

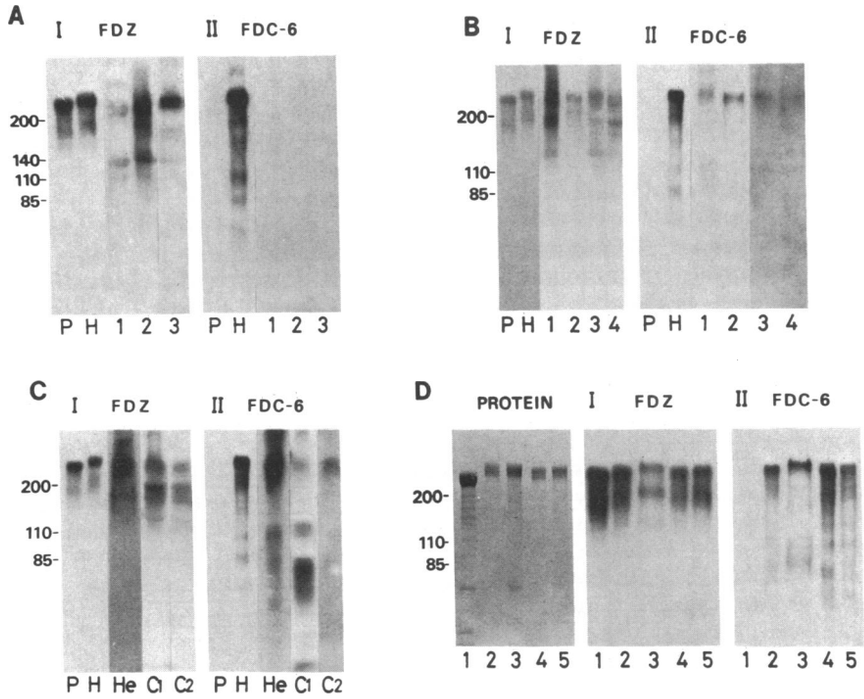

FIG. 3. Immunoblotting pattern of FNs extracted from human adult tissues (*A*), human fetal tissues (*B*), hepatomas and colon adenocarcinomas (*C*), and various cell lines from fetal tissue and hepatomas (*D*). In *A–D*, group I was blotted with monoclonal antibody FDZ, and group II was blotted with monoclonal antibody FDC-6. Molecular weights are shown as $M_r \times 10^{-3}$. (*A*) Group I: lane P, plasma FN; lane H, hepatoma FN from culture medium of HuH-7 cells. These two samples were used as reference. Note that lane P was not stained by FDC-6. Lane 1, skin FN; lane 2, paravertebral connective tissue FN; lane 3, large intestine FN. Note that no staining of those adult tissue FNs occurred with FDC-6. (*B*) Groups I and II: lanes P and H, the same as in *A*; lanes 1, FN from placenta; lanes 2, FN from amniotic fluid; lanes 3, FN from fetal connective tissue (carcass tissue mass from a 70-day embryo); lanes 4, FN from subcutaneous connective tissue of a 100-day fetus. (*C*) Groups I and II: lanes P and H, the same as in *A* and *B*; lanes He, FN from hepatoma tissue; lanes C1, FN from colon cancer case 1; lanes C2, FN from colon cancer case 2. These were both adenocarcinomas. The low molecular weight components in *C*, II, lanes He and Cl, were degradation products by an endogenous cathepsin-like protease, which was not inhibited by protease inhibitor added to the extract. (*D*) Groups I and II: lanes 1, plasma FN; lanes 2, FN from the culture medium of WI-38 fibroblasts; lanes 3, FN from the culture medium of VA13 sarcoma cells; lanes 4, FN from the culture medium of HuH-6 hepatoma cells; lanes 5, FN from the culture medium of HuH-7 hepatoma cells.

Absence of FN Reaction with the FDC-6 Antibody in Various Adult Tissues. FNs from adult human plasma, connective tissue (paravertebral region), and skin showed an obvious reactivity with the FDZ antibody, which defines a region in the cell domain (Fig. 3*A*, I), and these FNs had the same molecular weight as those of plasma. In contrast, none of these FNs from adult tissues and plasma reacted with monoclonal antibody FDC-6 (Fig. 3*A*, II, lanes P and 1–3). Extracts of normal adult spleen, kidney, and liver gave FN bands with similar reactivities as above—i.e., negative with FDC-6 and positive with the FDZ antibody (data not shown).

Reactivity of the FDC-6 Antibody with FNs from Placenta, Amniotic Fluid, Various Fetal Tissues, Tumor Tissues, and Culture Cells. FNs from placenta, amniotic fluid, fetal connective tissue, and fetal skin were reactive with the FDZ and FDC-6 antibodies (Fig. 3*B*, I and II). Extracts of some hepatomas and colon carcinoma tissues (Fig. 3*C*, I and II) as well as cell lines derived from fetal lung tissue (WI-38), hepatomas (HuH-6 and HuH-7), and sarcoma (VA13) (Fig. 3*D*, I and II) contained FN reactive with the FDZ and FDC-6 antibodies. However, some hepatoma and carcinomas did not contain FDC-6-reactive FN but contained FDC-6-negative, FDZ-positive FN (data not shown).

Quantitative Reactivity of FNs from Adult, Fetal, and Cancer Tissues. To quantitatively determine the reactivity of FNs from various sources with the FDC-6 antibody, the binding reactivity of the FDC-6 antibody per μg of FN was calculated as described in *Materials and Methods*. The results are shown in Fig. 4. As indicated, FNs from colon carcinoma, HuH-7 hepatoma, and placenta showed approx-

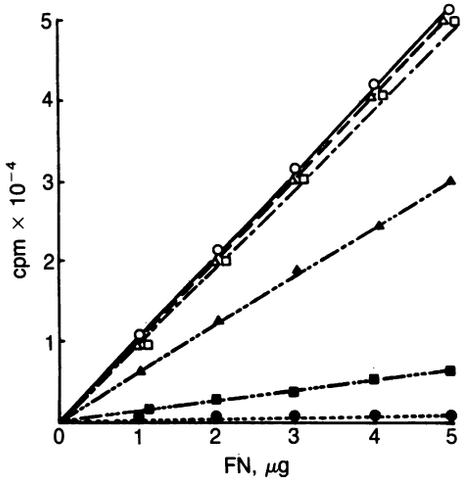

FIG. 4. Quantitative reactivity of various FNs with monoclonal antibody FDC-6. FNs isolated from culture media of HuH-7, plasma, and various tissue extracts were separated on $NaDodSO_4$/6.5% polyacrylamide gel electrophoresis followed by transfer onto nitrocellulose sheets. Multiple concentrations (1, 2, 3, 4, and 5 μg) of each purified FN were blotted with the FDZ antibody, and the blotted spots were assayed for radioactivity in a γ counter to determine the activity per μg. Another panel of multiple immunoblots of FN from each tissue with the FDC-6 antibody was made, and the FDC-6 blotting activity per μg of FN was determined. The ordinate indicates the binding activity of each FN with the FDC-6 antibody; the abscissa indicates the quantity of FN in μg determined by binding activity with the FDZ antibody. ○—○, HuH; ●- -●, plasma; △- - -△, placenta; □- - -□, colon; ▲- - -▲, fetal connective tissue; ■- - - -■, adult connective tissue.

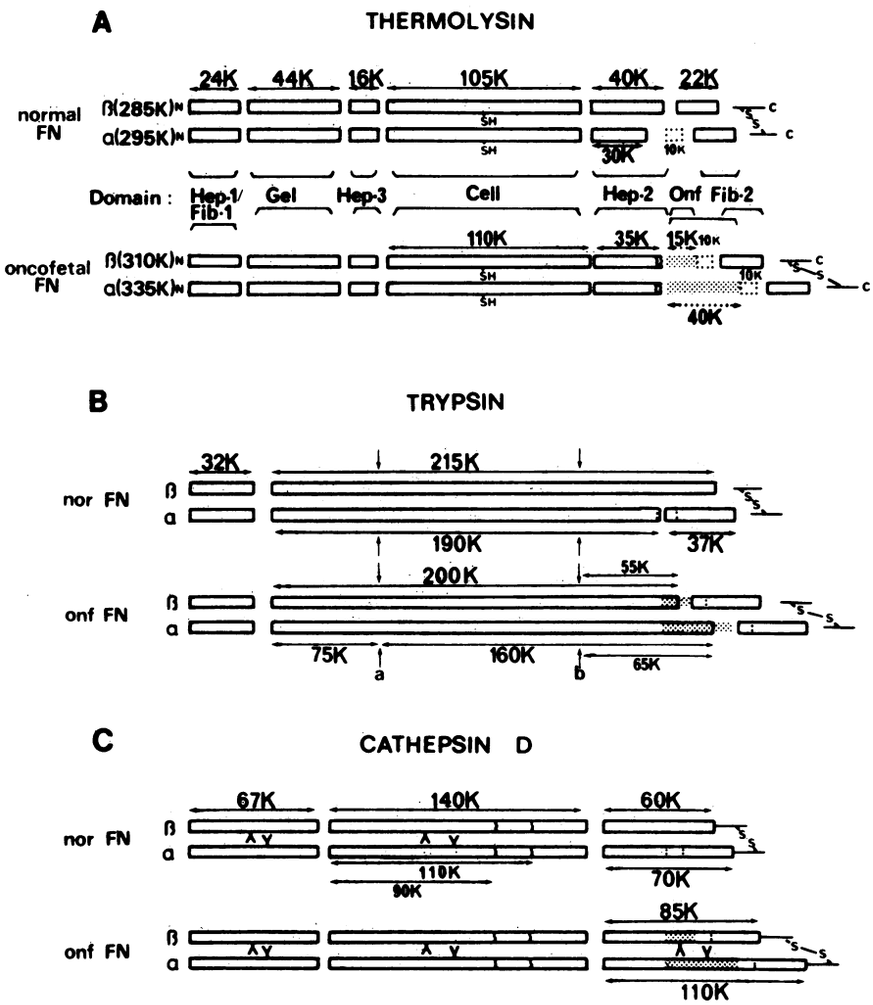

FIG. 5. Schematic drawing of FN domains released by limited proteolysis (modified from the previous model; refs. 16 and 17). Molecular weights are shown as $M_r \times 10^{-3}$. (*A*) Thermolysin releases six domains from nor FN: M_r 24,000 (Hep-1/Fib-1), M_r 44,000 (Gel), M_r 16,000 (Hep-3), M_r 105,000 (Cell), M_r 30,000–40,000 (Hep-2), and M_r 22,000 (Fib-2). The size of Hep-2 is M_r 30,000 from α and M_r 40,000 from β (16), and the α-chain may contain an undetectable M_r 10,000 domain based on calculation. Similar fragments were released by thermolysin from onf FN, except for the size of the Hep-2 domain (M_r 35,000, heterogenous). The location of the onf domain (indicated by shaded bar) is based on (*i*) the presence of FDC-6 reactivity at the COOH-terminal domain released by cathepsin D—i.e., positive in M_r 85,000–110,000 fragments from onf FN and negative in M_r 60,000–70,000 fragments from nor FN, as shown in *C*, and (*ii*) the absence of FDC-6 reactivity in the Hep-2 and Fib-2 domains released by thermolysin. The size of the onf domain is tentative and is based on calculation. (*B*) Trypsin digestion releases M_r 37,000 (containing Fib-2 domain), M_r 32,000 (Hep-1/Fib-1), and large M_r 215,000–190,000 fragments from nor FN, and a M_r 200,000 fragment from onf FN. Only the M_r 200,000 fragment from onf FN and no fragments from nor FN were stained by FDC-6. In addition, a strong staining was associated with smaller fragments (M_r 120,000–160,000 and M_r 55,000–65,000) derived from the middle domain by cleavage at points "a" and "b." Those fragments contained the onf domain. (*C*) Only the M_r 85,000 and M_r 110,000 fragments released by cathepsin D, which represent the COOH-terminal domain of onf FN, were stained with FDC-6. M_r 60,000 and M_r 70,000 fragments from nor FN were not stained.

imately the same range of binding activity to the FDC-6 antibody per μg of FN. The FN from fetal connective tissue showed a lower but obvious activity, whereas FNs from adult connective tissue and plasma showed barely detectable binding activity.

DISCUSSION

The results of this study clearly indicate two major findings. (*i*) There is a previously unrecognized FN domain or region defined by the monoclonal antibody FDC-6. The domain could be located between the previously known Hep-2 and Fib-2 domains (15, 16), as illustrated in Fig. 5, based on the data presented under *Results*.

(*ii*) There are two classes of human FNs. One contains the domain reactive with the FDC-6 antibody and has a large

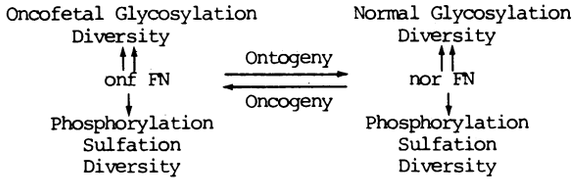

FIG. 6. Diversity of FNs as related to ontogenesis and oncogenesis. Deletion or insertion of the *oncofetal domain* in FN is associated with ontogenesis or oncogenesis (or both). The mechanism may essentially be based on differential splicing of a FN gene. A great deal of glycosylation diversity in FN has been found to be dependent on fetal or transformed cell origin (30–32). Heterogeneity of FN caused by phosphorylation or sulfation (33, 34) may also vary with oncogenic transformation. Thus, various posttranslational modifications may be essentially based on the presence or absence of the oncofetal domain.

molecular weight (subunit M_r, 310,000–335,000). This class is found in hepatomas and their cell lines, carcinomas and sarcomas and their cell lines, fetal connective tissue, fetal skin, fetal WI-38 fibroblasts, amniotic fluid, and placenta. Therefore, the FDC-6-defined domain is called "*oncofetal domain*," and FNs comprising the oncofetal domain are hereby referred to as "*oncofetal FN*" (onf FN) (see Fig. 5). The second class of FN is characterized by a smaller molecular weight and negative reactivity with the monoclonal antibody FDC-6. Plasma FN and FNs extracted from normal adult tissues such as connective tissue, skin, colon, liver, spleen, and kidney belong to this class of FN and are similar to the previously known plasma-form FN; they are hereby called "*normal FN*" (nor FN). The nor FN consists of α- and β-subunits with M_rs of 295,000 and 285,000, respectively (see Fig. 5). Previously, Ruoslahti *et al.* (29) observed a similarity in molecular weight and isoelectric point between FNs from human germ cell tumors and amniotic fluid and a distinctive difference from FN of plasma and suggested that such FNs may provide oncodevelopmental markers.

The FDC-6 antibody may not recognize a carbohydrate epitope based on the following findings: (*i*) its reactivity was resistant to sodium periodate/borohydride on solid phase, a method established to diagnose whether or not an epitope is carbohydrate (28), and (*ii*) onf FN did not react with various anticarbohydrate antibodies that define oncofetal carbohydrate determinants (unpublished observation).

These results suggest that switching from onf FN synthesis to nor FN synthesis is associated with human ontogenesis, and the reverse process is associated with oncogenesis (see Fig. 6). It is possible that undifferentiated, actively growing cells or tissues may synthesize onf FN. Interestingly, three of five cases of hepatoma, two of five cases of colonic adenocarcinoma, and two of four hepatoma cell lines produced onf FN in high quantities. However, two of five cases of hepatoma did not produce onf FN but produced nor FN. Therefore, oncogenic transformation may be associated with three different types of changes in FN synthesis: (*i*) a block in synthesis of either onf FN or nor FN, (*ii*) a switching of synthesis to onf FN from nor FN, and (*iii*) a continuous synthesis of nor FN, although the rate of synthesis is greatly decreased. A switching to onf FN synthesis may also be associated with selection of "normal" fibroblasts in a continuous culture—i.e., only those cells capable of synthesizing onf FN could survive as cultured cells. The synthesis of onf FN may well be a result of retrogenetic activation of "oncofetal" genes encoding the *oncofetal domain* defined by the FDC-6 antibody. Previously, two or three FN mRNAs were shown to exist in human Hs 578T tumor cells (35, 36) or in normal rat liver (37), respectively, but they are all derived from the transcript of a single gene (35–38). Variations in splicing a single FN gene may result in multiple subunits of FNs (35, 39). An apparent activation of the "oncofetal gene" encoding the onf domain may well be a consequence of splicing variations of a single FN gene encoding the COOH-terminal region. Determination of a functional role of the oncofetal domain in onf FN is of great interest and awaits further investigation.

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