Species-specific monoclonal antibodies in the assignment of the gene for human fibronectin to chromosome 2

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Using three different species-specific monoclonal antibodies we have studied, in human-mouse and human-hamster somatic cell hybrids, the correlation between the presence of different human chromosomes and the ability to release human fibronectin into the tissue culture medium. Presence of human fibronectin was determined by an affinity-radioimmunoassay. In addition, tissue culture media of the different hybrids were separated on SDS-polyacrylamide gels, the proteins were blotted onto a nitrocellulose sheet and human fibronectin visualized by an immunoenzymatic technique. Karyology and determination of isoenzyme markers of specific human chromosomes show that the ability to produce human fibronectin segregated with the presence of human chromosome 2.

Key words: blotting of proteins/somatic cell hybrids/immunoenzymatic staining of proteins

Introduction

The name fibronectin is used for a family of structural and immunologically related high mol. wt. glycoproteins that are present in vertebrates in both the soluble and insoluble forms (Mosesson and Amrani, 1980; Ruoslahti *et al.*, 1981; Yamada and Olden, 1978; Vaheri and Alitalo, 1981; Mosher, 1980; Vaheri and Mosher, 1978; Hynes, 1976; Grinnel, 1978). The soluble form has been observed in many different biological fluids including plasma, urine, cerebrospinal, and amniotic fluids. The insoluble form is present in vertebrate tissues, intracellularly and as an extracellular matrix component. The two different forms of fibronectin are immunologically similar and share many biological activities although they differ in several properties.

Although fibronectin's functions *in vivo* are not yet known, *in vitro* fibronectin presents many different interesting aspects. The amount of fibronectin present on the surface of transformed or neoplastic cells is generally greatly reduced with respect to their normal counterparts. Fibronectin has also been shown to promote cellular aggregation and cell adhesion to substrates, partially restore transformed fibroblasts to a more normal phenotype, promote locomotion of certain types of cells, and facilitate reticuloendothelial system clearance of particles. Fibronectin *in vitro* binds specifically to macromolecules such as collagen, fibrin and fibrinogen, heparin, actin, and DNA.

The genetic mechanisms whereby fibronectin expression is controlled are poorly understood. A better understanding of

these mechanisms would be of great value in developmental biology and oncology. Rodent-human cell hybrids have been extensively used to map the human genome (McKusick and Ruddle, 1977). These hybrid cell lines lose human chromosomes, and cloned hybrid cells can be obtained carrying different complements of human chromosomes. Linkage between the expression of human gene products and human chromosomes may be established in different hybrid cell strains and used to map the chromosomal location of human genes coding for particular proteins.

The chromosomal localization of the gene directing the synthesis of human fibronectin has recently been studied in different laboratories (Owerbach *et al.*, 1978; Eun and Klinger, 1980; Klinger and Ruoslahti, 1980; Rennard *et al.*, 1981). The difficulties in producing truly species-specific antibodies for the identification of human fibronectin could be one of the explanations for the contradictory results reported so far.

We have recently produced monoclonal antibodies to human fibronectin that do not cross-react with bovine, hamster, or mouse fibronectin (Zardi *et al.*, 1980a). Using these antibodies, we show here that in human-mouse and human-hamster somatic cell hybrids production of human fibronectin is concordant with the presence of human chromosome 2.

Results

Characterization of monoclonal antibodies against fibronectin

From our monoclonal antibodies against human fibronectin (Zardi *et al.*, 1980a) we have selected three that do not show any cross-reaction with mouse, bovine, or hamster fibronectin and are able to recognize different antigenic determinants on the human fibronectin molecule.

Species-specificity was tested by solid-phase radioimmunoassay using polyvinyl wells coated with purified human, mouse, bovine, and hamster fibronectin. A positive reaction was observed only using human fibronectin (data not shown).

Similar results were obtained using the affinity-radioimmunoassay as described in Materials and methods where the polyvinyl wells were first coated with gelatin and then incubated with fresh tissue culture medium or spent medium of parental cell cultures (Table I). Another requirement for the monoclonal antibodies selected was their ability to recognize different antigenic determinants, which would provide more information on the integrity of the human fibronectin molecules released from the different human-rodent somatic cell hybrids. The specificity of the monoclonal antibodies against different antigenic determinants was studied using the electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets followed by immunoenzymatic recognition of the fragments reacting with the different monoclonals.

Figure 1A shows a SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of fibronectin digested with different amounts of trypsin and stained with Coomassie brilliant blue. Figure

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Table I. Species-specificity of monoclonal antibodies to human fibronectin

Monoclonal antibody	Affinity-radioimmunoassay (c.p.m.)			
	Fresh medium	Spent medium ^a from mouse fibroblasts	Spent medium ^a from hamster fibroblasts	Spent medium from human fibroblasts
Clone 1	80	90	60	9550
Clone 2	70	70	40	7320
Clone 3	65	110	100	1580

^aSpent media from mouse and hamster fibroblasts were shown to contain homologous fibronectin as detected by electroimmunoassay.

Fig. 1. (A) SDS-PAGE of fibronectin digested with different amounts of trypsin stained with Coomassie brilliant blue. **Lane 1:** undigested fibronectin. **Lanes 2–6:** fibronectin digested with 0.1, 1.0, 10, 100, 640 µg/ml of trypsin. **Lane 7:** mol. wt. standards (Pharmacia, Uppsala, Sweden), rabbit muscle phosphorylase-b 94 000 daltons, BSA 67 000 daltons, egg white ovalbumin 43 000 daltons, bovine erythrocyte carbonic anhydrase 30 000 daltons, soybean trypsin inhibitor 20 100 daltons, bovine milk lactalbumin 14 400 daltons. (B) Nitrocellulose sheet on which proteins were transferred from a SDS-PAGE and stained by the heparin-toluidine procedure. **Lanes 1–7** as in A. (C–D) Two nitrocellulose sheets identical to the one shown in B after destaining and incubation with two different monoclonal antibodies followed by immunoenzymatic localization of fibronectin fragments reacting with two different monoclonal antibodies. For the procedures see Materials and methods.

1B shows a nitrocellulose sheet on which the fibronectin tryptic digest was transferred and stained by the heparin-toluidine procedure cited in Materials and methods. Figures 1C and D show two sheets identical to the one shown in Figure 1B after destaining and incubation with two different monoclonal antibodies followed by immunoenzymatic localization of the fibronectin fragments reacting with the monoclonal antibodies. The two monoclonal antibodies (Clone 1 and Clone 2) recognize different fragments indicating specificity for different antigenic determinants. All three monoclonal antibodies selected, showed different patterns indicating that our monoclonal antibodies are able to recognize three different antigenic determinants on the fibronectin molecule.

Fig. 2. Human fibronectin identification in the tissue culture media of: (1) LN human fibroblasts; (2) PAF x Balb IV-5 hybrid cell line; (3) NPAF x Balb-Va3-(2) hybrid cell line; (4) mouse fibroblasts. Tissue culture media were first separated on a 8% SDS-PAGE, proteins blotted onto nitrocellulose sheets, and human fibronectin visualized using species-specific monoclonal antibodies and the immunoenzymatic technique described in Materials and methods.

Correlation between expression of human fibronectin and human chromosome 2

We have studied the correlation between the ability of somatic cell hybrids to release human fibronectin into the tissue culture medium and the presence of different human chromosomes. Table II shows that the ability to produce human fibronectin segregated with the presence of human chromosome 2. The various isoenzyme chromosome markers were determined as cited in Materials and methods, and in all cases the cytogenetic analysis confirmed the biochemical findings. The presence of human fibronectin in tissue culture medium of the various hybrids was detected by the affinity-radioimmunoassay using gelatin-coated polyvinyl wells as described in Materials and methods. In all cases the three different monoclonal antibodies gave identical results.

Furthermore, human fibronectin in the tissue culture medium of the different hybrids was visualized using the blotting technique. Tissue culture media of hybrids were separated on 8% SDS-PAGE; the proteins were electrophoretically blotted onto a nitrocellulose sheet and human fibronectin visualized using species-specific monoclonal antibodies and the immunoenzymatic technique cited in Materials and methods (Figure 2). The results obtained with the affinity-radioimmunoassay agreed with those obtained using the blotting technique.

The assignment of the human fibronectin gene to chromosome 2 was confirmed by using a panel of five hybrid clones

Table II. Segregation of human fibronectin with enzyme markers in human-mouse and human-hamster cell hybrids

Isoenzyme marker symbol	Isoenzyme marker	Chromosome	No. of hybrid clones that are				Total clones tested	Percentage discordances
			+/+	-/+	+/-	-/-		
PGM1	Phosphoglucosmutase 1	1	2	4	1	17	24	21
IDH1	Isocitrate dehydrogenase (soluble)	2	15	1	0	18	34	3
GPX1	Glutathione peroxidase 1	3	2	5	0	9	16	31
PGM2	Phosphoglucosmutase 2	4	2	8	1	13	24	38
HEXB	Hexosaminidase-B (beta subunit)	5	4	6	5	9	24	46
SOD2	Superoxide dismutase (mitochondrial)	6	3	8	7	6	24	63
GUSB	Beta-glucuronidase	7	0	6	2	9	17	47
GSR	Glutathione reductase	8	8	5	5	13	31	32
ACO1	Aconitase (soluble)	9	1	7	1	7	16	50
GOT1	Glutamic-oxalacetic transaminase (soluble)	10	1	2	7	14	24	37
LDHA	Lactate dehydrogenase A	11	7	4	13	7	31	55
LDHB	Lactate dehydrogenase B	12	10	13	3	4	30	53
ESD	Esterase D	13	0	2	6	10	18	44
NP	Nucleoside phosphorylase	14	2	4	6	10	22	45
HEXA	Hexosaminidase-A (alpha subunit)	15	1	1	7	14	23	35
APRT	Adenine phosphoribosyltransferase	16	0	4	8	7	19	63
GALK	Galactokinase	17	1	6	8	9	24	58
PEPA	Peptidase A	18	1	0	7	11	19	37
GPI	Glucose phosphate isomerase	19	7	11	1	4	23	52
ADA	Adenosine deaminase	20	1	5	8	10	24	54
SOD1	Superoxide dismutase (soluble)	21	2	6	7	9	24	54
ACO2	Aconitase (mitochondrial)	22	0	7	2	7	16	56
G6PD	Glucose-6-phosphate dehydrogenase	X	4	3	5	12	24	33

Hybrid cells were studied for the expression of isoenzyme markers assigned to each of the different human chromosomes by starch gel or cellulose acetate gel electrophoresis. +/+, clones that both produce human fibronectin and carry the numbered chromosomes; -/+, clones that do not express human fibronectin but carry the numbered chromosomes; +/-, clones that produce human fibronectin and do not carry the numbered chromosomes; -/-, clones that neither express human fibronectin nor carry the numbered chromosomes.

Table III. Incidence of human chromosomes in human-mouse hybrids correlated with the production of human fibronectin

Hybrids	Human chromosomes (percentage of karyotyped cells containing the chromosome)																						Human fibronectin		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y	
PAF x Balb IV-5		100		20		10	50																30		+
NPAF x Balb Va3 (2) ^a	13	82					26													13					+
PAF x Balb IV-6		100		20		10	50																20		+
P x B II 12		100					90																		+

^aThis hybrid was recovered from a tumor obtained by injection of a hybrid cell line into nude mice.

(group three, see Materials and methods) containing almost only the human chromosome 2. Table III shows that these clones were positive for human fibronectin.

Discussion

To localize the human fibronectin gene we have analyzed the conditioned medium of human-mouse and human-hamster cell hybrids by affinity-radioimmunoassay using a panel of species-specific monoclonal antibodies to human fibronectin. Our results indicate that the human fibronectin gene is located on chromosome 2. We have also determined the mol. wt. of the antigen reacting in the affinity-radioimmunoassay. This was to exclude the possibility that our antibodies were reacting only with some human fibronectin fragments. The results obtained using the transfer technique for SDS-PAGE to nitrocellulose sheets indicate that all the monoclonal antibodies used recognize a protein from the tissue culture medium of the cell hybrids with a mol. wt. iden-

tical to that of fibronectin released by the human parental cell lines.

Localization of the human fibronectin gene on chromosome 8 (Owerbach *et al.*, 1978; Rennard *et al.*, 1981) and 11 (Eun and Klinger, 1980; Klinger and Ruoslahti, 1980) have previously been reported. In two of these reports (Owerbach *et al.*, 1978; Eun and Klinger, 1980), the technique used for the identification of fibronectin was unable to distinguish between human and mouse fibronectin. Owerbach *et al.* (1978) used the technique of labeling external membrane protein by lactoperoxidase-catalyzed iodination followed by identification of fibronectin on the basis of mol. wt. determined by SDS-PAGE. With these procedures it was not possible to distinguish between human and mouse fibronectin since it is known that mouse and human fibronectin have identical mol. wts. Eun and Klinger (1980) used an antiserum obtained by injecting rabbits with a mixture of mouse and rat fibronectin. This antiserum was obviously reacting strongly with mouse fibronectin. The necessity of using an assay able to

Fig. 3. (A) Metaphase of the human-mouse somatic cell hybrid clone PAF x Balb IV-5. (B) Karyotype of the metaphase shown in A. The arrows indicate the human chromosome 2.

discriminate between human and mouse fibronectin is of the utmost importance since it has been shown that in hybrids obtained from fibronectin-nonexpressing mouse cells and human cells the mouse fibronectin gene is often activated (Zardi *et al.*, 1979a; Klinger and Ruoslahti, 1980). In three of these studies cell hybrids were examined for the presence of human fibronectin only on the cell surface (Owerbach *et al.*, 1978; Klinger and Ruoslahti, 1980; Eun and Klinger, 1980). It is known that cells can synthesize fibronectin without retaining it on cell surface.

These points, plus difficulties in obtaining truly species-specific rabbit antibodies (Rennard *et al.*, 1981), may be the reason for discrepancies between our data and the above-mentioned reports. Clearly, additional information is needed to determine whether there are other loci involved in the expression of fibronectin.

The use of species-specific monoclonal antibodies for human fibronectin, together with the affinity-radioimmunoassay system and the transfer of proteins from SDS-PAGE to nitrocellulose sheets described here, should prove to be powerful tools in the study of fibronectin genetics.

Materials and methods

Hybrid cells

Three different groups of interspecific hybrids were studied. The first group of hybrid cells was produced by fusing Balb/c mouse peritoneal macrophages (MPM) with human 4MS4Va, PAF, and LN-SV cells (SV40-transformed human fibroblasts) (Croce *et al.*, 1974; Croce, 1977) in the presence of inactivated Sendai virus. The hybrids were selected in hypoxanthine-aminopterin-thymidine (HAT) medium (Littlefield, 1964) or medium containing 10^{-5} M ouabain (Croce, 1977). The second group of cell hybrids was produced by fusing a hypoxanthine-guanine-phosphoribosyltransferase (HGPRT)-deficient Chinese hamster cell line (Ng3) with normal human fibroblasts. The fused cultures were selected in HAT medium or in HAT medium supplemented with ouabain.

On the basis of the results obtained with these two groups of cell hybrids, we confirmed the localization of the human fibronectin gene using a third group of hybrid cells between mouse peritoneal macrophages and PAF SV40-transformed human cells that contained the complete mouse genome and very few human chromosomes including chromosome 2 (Table III and Figure 3). Hybrids of this group were either cultured *in vitro* for >30 passages or derived from tumors induced in nude mice by the injection of hybrid cells. With this procedure, cell hybrids containing the SV40 genome were selected (Littlefield, 1964; Croce and Koprowski, 1974a; Croce *et al.*, 1975).

The details of the procedures used for cell culture and cell cloning, subcloning, and selection are described elsewhere (Croce *et al.*, 1973a; Croce and Koprowski, 1974b; Croce *et al.*, 1974; Croce, 1977). All the parental cell lines produced homologous fibronectin and released it into the medium as determined by electroimmunoassay using rabbit antibodies to human or mouse fibronectin (Zardi *et al.*, 1979a 1980b).

Karyologic and isoenzyme analysis

Metaphase chromosomes of hybrid cells were banded according to a modification of the trypsin Giemsa method of Seabright (1971) (Croce *et al.*, 1973b). At least 25 metaphases were karyotyped for each hybrid cell line.

Both human-hamster and human-mouse hybrid cells were studied for the expression of isoenzyme markers assigned to each of the different human chromosomes by starch gel or cellulose acetate electrophoresis as described by O'Brien *et al.* (1978). Karyologic and isoenzyme marker analysis on the hybrid cell lines was carried out contemporaneously to the fibronectin assay.

Monoclonal antibodies and radioimmunoassay

Anti-human plasma fibronectin monoclonal antibodies were prepared as previously described (Zardi *et al.*, 1980a). To test the species-specificity of antibodies we have used a modification of the previously described solid-phase double antibody radioimmunoassay using purified fibronectin from human, mouse, calf, or hamster serum as antigen (Zardi *et al.*, 1980a; Zardi *et al.*, 1979b; Klinman *et al.*, 1976).

We used an affinity-radioimmunoassay to detect the presence of human fibronectin in the tissue culture medium of rodent-human somatic cell hybrids. The polyvinyl wells were first coated with gelatin, incubating 100 μ l of a solution containing 1 mg/ml of Bacto gelatin (DIFCO Labs., Detroit, MI) in 0.15 M NaCl 20 mM phosphate buffered saline (PBS), pH 7.2 for 2 h.

After washing with PBS and saturation of protein binding sites by a solution of 1% bovine serum albumin (BSA) in PBS, the wells were incubated with the tissue culture medium of the various hybrid cell lines. Under these conditions only fibronectin can bind to the gelatin coating the wells. Presence of human fibronectin was subsequently detected using species-specific monoclonal antibodies and [125 I]rabbit anti-mouse Fab as previously described (Zardi *et al.*, 1980a).

Transfer of protein from SDS-PAGE to nitrocellulose sheets and reversible staining of the proteins by heparin-toluidine

SDS-PAGE was performed according to Laemmli (1970) using vertical slab gels. The transfer of protein from SDS-PAGE to nitrocellulose sheets was performed as previously described by Towbin *et al.* (1979). Heparin-toluidine blue staining was used to locate transferred proteins on the nitrocellulose sheets (Vartio and Vaheri, 1981). This reversible staining procedure gives a pattern similar to that obtained with Coomassie brilliant blue R250 and the same nitrocellulose sheet, after destaining, can be used for immunoenzymatic studies (Towbin, personal communication).

Fibronectin and fibronectin fragments were localized in the nitrocellulose sheet using monoclonal antibodies to human plasma fibronectin as described by Towbin *et al.* (1979) using peroxidase-conjugated rabbit anti-mouse Ig as second antibody (DAKO, Copenhagen, Denmark).

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