Modulation by extracellular matrices of monooxygenase and CYP1A1 induction in Hep G2 cells in serum-free culture

Akihiko Nakama

Osaka City Institute of Public Health and Environmental Sciences, 8-34 Tojo-cho, Tennoji-ku, Osaka 543-0026, Japan

Received 14 May 1998; accepted 7 January 1999

Key words: cytochrome P4501A1, extracellular matrices, fibronectin, Hep G2, mixed-function monooxygenase, serum-free culture

Abstract

The *in vitro* cellular functions of differentiated cells are influenced by culture conditions. Effects of several extracellular matrices (ECMs) on cytochrome P450-dependent monooxygenases (MFOs) induction and cytochrome P4501A1 (CYP1A1) gene expression were estimated in Hep G2 cells cultured in a serum-free medium. The cells were cultured on collagen type I- and II-, fibronectin-, and matrigel-coated dishes and MFO activities were induced by the addition of 3-methylcholanthrene (MC). The induction of ethoxycoumarin *O*-deethylase (ECOD) and alkoxyresorufin *O*-dealkylase activities as well as the expression of CYP1A1 mRNA were also determined. ECOD and methoxy- and ethoxyresorufin *O*-dealkylase activities in Hep G2 cells were enhanced by culturing the cells using a serum-free medium on fibronectin- or matrigel-coated dishes. ECOD activity on fibronectin-coated dishes was about 3-fold higher than that using a serum-supplemented medium on untreated dishes. Furthermore, both immobilized and soluble fibronectin enhanced the induction of MFOs. The expression of CYP1A1 mRNA using fibronectin-coated dishes was about 2-fold higher than that using a serum-supplemented medium on untreated dishes. These findings suggest that the gene expression in cultured cells is greatly influenced by ECMs. By using fibronectin-coated dishes to cell culture in a serum-free medium, reproducible and highly sensitive results can be obtained in experiments using cultured cells.

Abbreviations: ECMs, extracellular matrices; MFO, cytochrome P450-dependent mixed-function monooxygenase; PAHs, polycyclic aromatic hydrocarbons; FBS, fetal bovine serum; DMSO, dimethylsulfoxide; MC, 3-methylcholanthrene; PB, sodium phenobarbital; ECOD, 7-ethoxycoumarin *O*-deethylase; AROD, 7-alkoxyresorufin *O*-dealkylase; MROD, 7-methoxyresorufin *O*-demethylase; EROD, 7-ethoxyresorufin *O*-deethylase; PROD, 7-pentoxyresorufin *O*-depentylase; BROD, 7-benzyloxyresorufin *O*-debenzylase

Introduction

Hep G2 is a highly differentiated human hepatoma cell line, and is known to retain various cellular functions that are often lost during cell culture (Knowles et al., 1980; Campart et al., 1989). This cell line possesses enzyme activities relating to phase I (cytochrome P450-dependent monooxygenase, MFOs) and phase II (glucuronate and sulfate conjugation) metabolism of xenobiotics. Thus, the cell line has been utilized in *in vitro* experiments for investigating the

metabolism and toxicity of drugs instead of human normal hepatocytes (Doostdar et al., 1991; Patel et al., 1991; Grant et al., 1988). However, it has been well documented that MFO activities in Hep G2 cells are affected by the composition of the culture medium (Doostdar et al., 1988). When Hep G2 cells are cultured in a regular medium supplemented with serum, it is difficult to evaluate the effects of various serum components such as extracellular matrices (ECMs) or hormones on cellular metabolism. Therefore, to eliminate such a problem, Hep G2 cells must be cultured

in a serum-free medium supplemented with growth factors. Nakama et al. (1995) demonstrated that the induction of 7-ethoxyresorufin *O*-deethylase (ECOD) activity by 3-methylcholanthrene (MC) in Hep G2 cells cultured using a serum-free medium on collagen type I-coated dishes was about twice as high as that in the cells cultured in a medium supplemented with serum. In addition, ECMs were essential for the adhesion of Hep G2 cells, when the serum-free medium was used.

ECMs are known to possess various biological functions including morphogenesis, wound healing, and metastasis (Hedin and Thyberg, 1987; Kleinman et al., 1987; Linask and Lash 1988; Martin and Timpl, 1987). Fibronectin is one of the best-characterized adhesive glycoproteins. The biological roles of fibronectin include not only the adhesion of cell to ECM, but also erythrocyte coagulation, cell extension, cell differentiation, and cell proliferation through VLA-5 fibronectin receptor which belongs to the integrin gene family (Yamada, 1989). Fibronectin is known to be essential, as a growth factor, for culturing cells such as rat follicle cell line, RF-1 and mouse embryonic carcinoma, F₉ (Orly and Sato, 1979; Rizzino and Crowley, 1980).

To evaluate the effects of environmental chemicals on human health, MFO activities induced *in vitro* by these contaminants are used as a biomarker (Nakama et al., 1997). However, due to the small amount of samples obtained as environmental contaminants, it is essential to have a stable and highly sensitive method to measure MFO activities induced *in vitro*. In the study presented here, the effects of ECMs on the *in vitro* induction of MFO activity and the expression of cytochrome P450 gene using Hep G2 cells cultured in a serum-free medium.

Materials and methods

Materials

Collagen type I solution (3 mg/ml: porcine achilles tendon) and collagen type II solution (3 mg/ml: calf cartilage) were obtained from Koken, Inc. (Tokyo, Japan). Matrigel was purchased from Becton Dickinson (Bedford, MA, U.S.A.), and gelatin-Sepharose from Bio-Rad (Richmond, CA, U.S.A.). Fibronectin was prepared from porcine plasma using gelatin-affinity chromatography (Loo et al., 1989). Methoxy-, ethoxy-, pentoxy-, and benzyloxyresorufin were synthe-

ized as described by Mayer et al. (1977). All other reagents were of reagent grade.

Cell culture and enzyme induction

The human hepatocellular carcinoma cell line, Hep G2 (ATCC HB 8065), at passage 76 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.). The cells were maintained in a serum-free medium supplemented with growth factors in a humidified 5% CO₂-95% air atmosphere at 37 °C as previously described (Nakama and Yamada, 1993). A mixture of Dulbecco and Vogt's modification of Eagle's medium and Ham's F-12 (1:1) were used as basal medium. 10 µg/ml Insulin, 10 µg/ml transferrin, 30 nM sodium selenite, 10 ng/ml epidermal growth factor, 0.2 mM L-ascorbic acid phosphate magnesium salt, 50 nM dexamethasone, 10 µM o-phosphorylethanolamine, 1 nM triiodo-L-thyronine, and 100 µg/ml egg yolk low density lipoprotein were added to basal medium as growth factors. The cells were also independently cultured in a medium containing 10% (V/V) fetal bovine serum without the addition of growth factors. The cells were plated on ECM-coated culture dishes (diameter, 60 mm) at a density of about 1×10^6 cells/dish. When the cells reached confluence, fresh medium was added and the cells were exposed to MFO inducers (MC) dissolved in DMSO for 16 h before each experiment. DMSO at the final concentration of 0.2% in the medium did not affect the induction of the enzyme.

ECM coating of dish surface

Collagen and matrigel were diluted to 100~300 µg/ml with 1 mM HCl and to 300 µg/ml with cold basal medium, respectively. Fibronectin was diluted to a final concentration of 10 µg/ml with phosphate-buffered saline. The 60 mm dishes were exposed to 2 ml collagen or matrigel for 30~60 min, or to 2 ml fibronectin for 1 h at 37 °C, and after discarding these solutions, the cells were cultured in a fresh medium.

Enzyme assays

ECOD and AROD activities were measured as described by Nakama et al. (1995). The enzyme activity is expressed as nanomole per milligram of protein per 3 h.

Northern blot analysis

Total RNA was isolated from the cells cultured on a dish (diameter, 100 mm), and was extracted according to the method described by Chomczynski and Sacchi (1987). The RNA samples (20 μ g) were resolved on 1% agarose gels containing formaldehyde and capillary transferred onto Amersham Hybond-N+ membranes (Buckinghamshire, England). Human CYP1A1 cDNA (pHP1-450-3') was obtained from ATCC. cDNA labeling and RNA detection were carried out using Amersham ECL direct nucleic acid labeling and detection systems (Amersham RPN 3000), and the emitted signal was captured by the exposure to Hyperfilm-ECL.

Statistic analysis

Statistic differences between treatment groups were evaluated by the analysis of variance using Tukey's comparison.

Results and discussion

Induction of ECOD activity in Hep G2 cells cultured on ECM-coated dishes

The activities of ECOD in Hep G2 cells that were cultured in serum-free medium for 7 days using dishes coated with four types of ECMs (collagen type I, collagen type II, fibronectin, and matrigel) followed by the treatment with 2.5 μ M MC for 16 h are shown in Figure 1. These activities were compared with those of control cells cultured in 10% FBS-supplemented medium on uncoated commercial dishes. On every ECM-coated dish, Hep G2 cells treated by MC showed higher ECOD activities (ranging from 7 to 14 nmol/mg protein/3 h) than intact cells (0.05~0.24 nmol/mg protein/3 h). The activities of ECOD in cells cultured on fibronectin- or matrigel-coated dishes were significantly higher than those in control cells. The mean activity of ECOD in cells cultured on fibronectin-coated dishes was about 3-fold increased as compared to that in control cells. The activity of ECOD in cells grown on collagen type I-coated dishes in serum-supplemented medium was similar to the control cell (data not shown), indicating that this ECM did not affect the induction of the enzyme and possessed only an adhesive function. Fibronectin and matrigel enhanced the induction of ECOD activities as compared to collagens I and II,

suggesting that these have functions other than adhesion to Hep G2 cells. Furthermore, the effects of these ECMs once established on the first day of culture lasted for 7 days.

Effect of fibronectin on the induction of ECOD in Hep G2 cells

Hep G2 cells were cultured in serum-free medium on collagen-coated dishes. The effect of the various amounts (μ g/dish) of fibronectin on ECOD induction with the presence and absence of 3-methylcholanthrene (2.5 μ M) is shown in Figure 2. ECOD activity in cells treated with 3-methylcholanthrene was enhanced as the amount of fibronectin increased, whereas the activity in intact cells unchanged. The addition of 150 μ g/dish fibronectin induced the same activity (14 nmol/mg protein/3 h, Figure 2) as fibronectin-coated dishes as shown in Figure 1. In order to maintain the effect of fibronectin, it was essential to add fibronectin to the medium, whenever fresh medium was used. Therefore, a large amount of fibronectin was required for such an experimentation. In order to obtain the same activity of enzyme using fibronectin, the use of fibronectin-coated dishes may be less laborious and more economical, since only the initial treatment of dishes with fibronectin is required.

Fukai et al. (1992) demonstrated that protein synthesis in mouse embryo fibroblastic (NIH-L1) cells was stimulated by the addition of fibronectin in solution (as a soluble factor) to the culture medium. However, they reported that the synthesis was not enhanced, when fibronectin-coated dishes were used for cell culture. Moreover, Li et al. (1997) reported that immobilized fibronectin enhanced lymphokine-activated killer (LAK) cell activity, whereas soluble form of fibronectin showed no effect on LAK activity or cell proliferation. Contrary to these reports by Fukai et al. (1992) and Li et al. (1997), our findings presented in this study indicate that both immobilized and soluble fibronectin enhanced the induction of CYP1A1 by MC using Hep G2 cells. Our data together with those of others suggested that the effect of fibronectin as well as how it is used depend on the type of cells under investigation.

Induction of AROD activities in Hep G2 cells on ECM-coated dishes

AROD activities in Hep G2 cells cultured on collagen type I- and fibronectin-coated dishes are shown

Figure 1. Effect of ECMs on the induction of ECOD activity in Hep G2 cells. Cells were incubated with the presence (Treatment) or absence (Intact) of 2.5 μ M 3-methylcholanthrene for 16 h under the conditions below. Collagen I, serum-free medium on collagen type I-coated dishes; Collagen II, serum-free medium on collagen type II-coated dishes; Fibronectin, serum-free medium on fibronectin-coated dishes; Matrigel, serum-free medium on matrigel-coated dishes; Control, 10% FBS-supplemented medium on untreated dishes. The results are expressed as means $\pm$ SD of four experiments. * Significantly different from Collagen II and Control, $P < 0.01$; Collagen I, $P < 0.05$. † Significantly different from Control, $P < 0.01$.

in Table 1. MROD activities in all intact, sodium phenobarbital- and MC-treated cells were enhanced by fibronectin coating on dishes, and increased 2~3-fold compared to collagen. EROD activity in MC-treated cells on fibronectin-coated dishes was about 2-fold higher than that cultured on collagen-coated dishes. Fibronectin, however, did not affect EROD activity in intact cells as compared to MROD. PROD activity was not affected with sodium phenobarbital- or MC-treatment, but fibronectin enhanced the potential PROD activity in the cells. BROD activity was affected with neither inducers nor fibronectin. These findings suggested that the effect of fibronectin depends on cellular functions.

CYP1A1 mRNA expression in Hep G2 cells on ECM-coated dishes

The expression of CYP1A1 mRNA was observed with Northern blotting (Figure 3). In every culture condition stated above, MC induced CYP1A1 mRNA. The

expression of mRNA in cells cultured on fibronectin-coated dishes was 1.9-fold larger than that in control cells as determined by an image analyzing system. Sodium phenobarbital also slightly induced CYP1A1 mRNA expression in the cells cultured on collagen- or fibronectin-coated dishes. Furthermore, it was found that CYP1A1 proteins were translated from CYP1A1 gene with western blotting where goat anti-rat CYP1A1 antibody (Daiichi Pure Chemicals Co., Tokyo, Japan) was used. Similar results were observed for Northern blotting (data not shown). Our findings suggested that fibronectin enhances the expression of CYP1A1 mRNA that is induced by exposure to PAHs, and as a consequence, ECOD and AROD activities are increased.

It is likely that the fibronectin-induced CYP1A1 activity is due to greater Hep G2 cell differentiation, because the contributions of fibronectin to cell differentiation have been shown in several studies (Peenypacker et al., 1979; Yamada et al., 1985; Ro-

Figure 2. Effect of fibronectin solution on the induction of EROD activity in Hep G2 cells. Cells were incubated with (○) and without (●) 2.5 μ M 3-methylcholanthrene for 16 h in a serum-free medium in collagen type I-coated dishes. Fibronectin was added to the medium as a soluble factor. The results are expressed as means $\pm$ SD of four experiments. * Significantly different from 0, 5, 10 μ g/dish of fibronectin, $P < 0.01$; 25 μ g/dish, $P < 0.05$.

Table 1. Induction of AROD activities in Hep G2 cells in collagen type I- and fibronectin-coated dishes

	Collagen type I			Fibronectin		
	Intact	PB	MC	Intact	PB	MC
(nmole/mg protein/3 h)						
MROD	1.60 $\pm$ 0.60	1.66 $\pm$ 0.50	4.10 $\pm$ 0.56	3.97 $\pm$ 0.32*	5.09 $\pm$ 0.76*	8.92 $\pm$ 1.31*
EROD	0.79 $\pm$ 0.44	1.14 $\pm$ 0.39	6.47 $\pm$ 1.22	0.46 $\pm$ 0.79	2.22 $\pm$ 0.51	12.68 $\pm$ 1.31*
PROD	0.10 $\pm$ 0.41	0.00 $\pm$ 0.32	0.07 $\pm$ 0.25	2.41 $\pm$ 1.42†	2.12 $\pm$ 0.77*	1.81 $\pm$ 0.45
BROD	0.71 $\pm$ 0.08	0.56 $\pm$ 0.11	0.88 $\pm$ 0.18	1.09 $\pm$ 0.28	1.01 $\pm$ 0.17	0.98 $\pm$ 0.16

MROD, 7-methoxyresorufin *O*-demethylase; EROD, 7-ethoxyresorufin *O*-deethylase; PROD, 7-pentoxymethoxyresorufin *O*-deethylase; BROD, 7-benzoyloxyresorufin *O*-debenzylase; Hep G2 cells were cultured in serum-free medium in collagen type I- and fibronectin-coated dishes, and incubated with 2 mM phenobarbital (PB) or 2.5 μ M 3-methylcholanthrene (MC) for 16 h. Results are the means $\pm$ SD of three experiments. Significantly different from collagen type I, $P < 0.01$ (*), $P < 0.05$ (†).

Figure 3. Northern blot analysis of CYP1A1 mRNA expressed in Hep G2 cells. Cells were incubated with 2 mM phenobarbital (PB) or 2.5 μ M 3-methylcholanthrene (MC) for 16 h. Total cellular RNA was extracted and analyzed by Northern blot hybridization, using a cDNA to human CYP1A1, as described in Materials and Methods. Control, using 10% FBS-supplemented medium in untreated dishes; COL, using a serum-free medium in collagen type I-coated dishes; FN, using a serum-free medium in fibronectin-coated dishes.

gers, 1990), and the growth of Hep G2 cells on fibronectin was slower than that on collagen type I (Nakama and Yamada, 1993). Furthermore, when the expression of oncogene c-myc and N-ras in Hep G2 cells was observed using the fluorescence antibody technique, the expression of N-ras was suppressed by the cells cultured on fibronectin-coated dishes (data not shown). The decrease in N-ras expression may indicate further differentiation in Hep G2 cells, and the enhanced CYP1A1 activity may represent the native activity of human hepatocytes. Matrigel enhanced the MFO activities slightly more in Hep G2 cells as compared to collagen type I, but the activities were lower than those in the cells cultured with fibronectin (Figure 1). Matrigel may be less effective to re-differentiate the dedifferentiated cells than fibronectin.

The MFO activity of rat hepatocytes in primary culture drastically decreases during cell culture (Grant et al., 1985). P450 expression or regulation is unstable in rodent normal hepatocytes. Furthermore, differences in cytochrome P450 between rodents and humans have been demonstrated (Gonzalez, 1989; Guengerich, 1992). There is a conservation of catalytic specificity among the human P450s and their animal orthologs. For instance, this appears to be the general

case in the P450 1A1, 1A2, and 2E1 protein families. However, apparent discrepancies are sometimes seen because another protein present in the animal tissue may have an unusually high activity toward a particular substrate such as nifedipine. Rodents have not only MC- but also PB-type induction, which involve the cytochrome P450 2B enzymes that play a major role in the metabolism of xenobiotics such as aflatoxin B₁. PB selectively induces hepatic P450 2B1 and P450 2B-catalyzed PROD activity in rats *in vivo* (Burke et al., 1985). But there is no convincing evidence in the literature that PB induces P450 2B in human *in vivo*, despite the presence of the CYP2B6 gene in human liver. Thus the effects of environmental contaminants upon human health cannot be precisely evaluated by extrapolating rodent data. Employing human cell lines such as Hep G2 should be useful for studying the effects of extensive environmental samples, such as extracts from drinking water, river water or suspended particles in air. They could also be used to evaluate the effects of environmental contaminants upon human health, and thus be applied to human risk assessment. Nakama et al. (1995) demonstrated that serum-free medium could provide reproducible and highly sensitive results since the cells are constantly cultured under the same conditions. Moreover, in this

study it was demonstrated that serum-free cultivation using fibronectin-coated dishes could provide highly sensitive results.

Fibronectin occurs in two different forms. The soluble plasma fibronectin is found in blood and other body fluids. An insoluble form, cellular fibronectin, is part of the extracellular matrix. In this study the soluble plasma fibronectin prepared from porcine plasma was used. Hereafter it will be necessary to evaluate the effects of cellular fibronectin or fibronectin prepared from other species on the cellular functions *in vitro*.

References

- Burke MD, Thompson S, Elcombe CR, Halpert J, Haaparanta T and Mayer RT (1985) Ethoxy-, pentoxy- and bezyloxy-phenoxanzones and homologues; a series of substrates to distinguish between different induced cytochromes P-450. *Biochem Pharmacol* 34: 3337–3345.
- Campart GB, Canonero R, Mereto E and Ferro M (1989) Cytotoxic and genotoxic effects of 10 N-nitroso compounds in a human hepatoma cell line (Hep G2): comparison with human hepatocyte primary cultures. *ATLA* 17: 22–27.
- Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156–159.
- Doostdar H, Burke MD, Melvin WT and Grant MH (1991) The effects of dimethyl-sulphoxide and 5-aminolaevulinic acid on the activities of cytochrome P450-dependent mixed function oxidase and UDP-glucuronosyltransferase activities in human Hep G2 hepatoma cells. *Biochem Pharmacol* 42: 1307–1313.
- Doostdar H, Duthie SJ, Burke MD, Melvin WT and Grant MH (1988) The influence of culture medium composition on drug metabolising enzyme activities of the human liver derived Hep G2 cell line. *FEBS Letter* 241: 15–18.
- Fukai F, Iso T and Katayama T (1992) Stimulation of the protein synthesis by fibronectin is specific for untransformed fibroblastic cells. *Biochem Int* 27: 511–516.
- Gonzalez FJ (1989) The molecular biology of cytochrome P-450s. *Pharmacol Rev* 40: 243–288.
- Grant MH, Burke MD, Hawksworth GM, Duthie SJ, Engeset J and Petrie JC (1987) Human adult hepatocytes in primary monolayer culture. Maintenance of mixed function oxidase and conjugation pathways of drug metabolism. *Biochem Pharmacol* 36: 2311–2316.
- Grant MH, Duthie SJ, Gray AG and Burke MD (1988) Mixed function oxidase and UDP-glucuronyltransferase activities in the human Hep G2 hepatoma cell line. *Biochem Pharmacol* 37: 4111–4116.
- Grant MH, Melvin MAL, Shaw P, Melvin WT and Burke MD (1985) Studies on the maintenance of cytochromes P450 and monooxygenase and cytochrome reductases in primary cultures of rat hepatocytes. *FEBS Lett* 190: 99–103.
- Guengerich FP (1992) Characterization of human cytochrome-P450 enzymes. *FASEB Journal* 6: 745–748.
- Hedin U and Thyberg J (1987) Plasma fibronectin promotes modulation of arterial smooth-muscle cells from contractile to synthetic phenotype. *Differentiation* 33: 239–246.
- Kleinman HK, Graf J, Iwamoto Y, Kitten GT, Ogle RC, Sasaki M, Yamada Y, Martin GR and Luckenbill-Edds L (1987) Role of basement membranes in cell differentiation. *Ann NY Acad Sci USA* 513: 134–145.
- Knowles BB, Howe CC and Aden DP (1980) Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science* 209: 497–499.
- Li YQ, Kobayashi M, Kuramitsu Y, Yuan L, Matsushita K, Yagita H, Okumura K and Hosokawa M (1997) Enhancement of lymphokine-activated killer cell activity by fibronectin. *J Immunotherapy* 20: 123–130.
- Linask KK and Lash JW (1988) A role for fibronectin in the migration of avian precardiac cells. II. Rotation of the heart-forming region during different stages and its effects. *Dev Biol* 129: 324–329.
- Loo D, Rawson C, Ernst T, Shirahata S and Barnes D (1989) Primary and multipassage culture of mouse embryo cells in serum-containing and serum-free media. In: Baserga R (ed) *Cell growth and division, A practical approach* (pp. 17–35) IRL Press, Oxford, England.
- Martin GR and Timpl R (1987) Laminin and other basement membrane components. *Ann Rev Cell Biol* 3: 57–85.
- Mayer RT, Jermyn JW, Burke MD and Prough RA (1977) Methoxyresufurin as a substrate for the fluorometric assay of insect microsomal O-dealkylases. *Pest Biochem Physiol* 7: 349–354.
- Nakama A, Kuroda K and Yamada A (1995) Induction of cytochrome P450-dependent monooxygenase in serum-free cultured Hep G2 cells. *Biochem Pharmacol* 50: 1407–1412.
- Nakama A and Yamada A (1993) Serum-free culture of human hepatoma Hep G2 cells with egg yolk low density lipoprotein. *Biosci Biotech Biochem* 57: 410–413.
- Nakama A, Yoshikura T and Fukunaga I (1997) Induction of cytochrome P450 in Hep G2 cells and mutagenicity of extracts of sediments from a waste disposal site near Osaka, Japan. *Bull Environ Contam Toxicol* 59: 344–351.
- Orly J and Sato G (1979) Fibronectin mediates cytokinesis and growth of rat follicular cells in serum-free medium. *Cell* 17: 295–305.
- Patel DK, Shockcor JP, Chang SY, Sigel CW and Huber B (1991) Metabolism of a novel antitumor agent, crisnatol, by hepatoma cell line, Hep G2, and hepatic microsomes. *Biochem Pharmacol* 42: 337–346.
- Rizzino A and Crowley C (1980) Growth and differentiation of embryonal carcinoma cell line F9 in defined media. *Proc Natl Acad Sci USA* 77: 457–461.
- Rogers SL (1990) Substratum effects on cell dispersal, morphology, and differentiation in cultures of avian neural crest cells. *Dev Biol* 141: 173–182.
- Yamada KM (1989) Fibronectins: structure, functions and receptors. *Curr Opin Cell Biol* 1: 956–963.
- Yamada KM, Akiyama SK, Hasegawa T, Hasegawa E, Humphries MJ, Kennedy DW, Nagata K, Urushihara H, Olden K and Chan WT (1985) Recent advances in research on fibronectin and other cell attachment proteins. *J Cell Biochem* 28: 79–97.