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Childhood kidney tumours: in vitro studies and natural history

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Summary. The growth patterns of mesoblastic nephroma, Wilms' tumour and bone metastasizing renal tumour of childhood (BMRTC) are distinct from one another and are therefore useful in distinguishing children's kidney tumours. Of the 3 tumour types only BMRTC was able to invade native collagen gels. Fibronectin was present in Wilms' tumour and mesoblastic nephroma, but absent from BMRTC. The readdition of fibronectin to fibronectin depleted tissue culture medium markedly reduced the ability of BMRTC to migrate into collagen gels. The significance of the results and their relevance to the natural history of these neoplasms is discussed.

Key words: Childhood kidney tumours – Fibronectin – Kidney tumours in tissue culture

Kidney tumours are one of the most important groups among the solid neoplasms of children. Of these nephroblastoma, or Wilms' tumour is the most frequent and was originally described as a mixed sarcomatous and epithelial tumour with blastemal elements. The presence of a large variety of connective tissue components give it a wide spectrum of histological appearances. Different variants of Wilms' tumours have been reported. For instance, rhabdomyomatous nephroblastoma contains at least 60% striated muscle cells and none or very few epithelial elements. Mesoblastic nephroma and cystic nephroblastoma have also been separated from Wilms' tumour because they are well differentiated and have an excellent prognosis. (Marsden and Steward 1976; Wigger 1976; Marsden et al. 1984; Beckwith 1984 and Stambolis 1984). During the past few years another kidney tumour has been separated from Wilms' tumour and is now regarded as a new entity and has a worse prognosis. This is bone-metastasizing renal tumour of childhood (BMRTC) (Marsden et al. 1978). There is evidence that

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Table 1. Age and incidence of mesoblastic nephroma, Wilms' tumour and bone metastasising renal tumour of childhood

Age (years)	Incidence of		
	Mesoblastic nephroma	Wilms'	BMRTC
Before full-term	2 (28 and 36 weeks)	0	0
0–1	10 (includes 2 neonates)	19 (youngest 2 months)	2 (both 10 months)
1–2	5 (oldest 21 months)	28	17
2–3	0	23	13
3–4	0	21	7
4–5	0	11	4
5–6	0	13	3
6–7	0	2	1
7–8	0	2	0
8–9	0	2	0
9–10	0	2	0
10–11	0	1	1
11–12	0	1	0
12–13	0	1	0
13–14	0	0	1
14–15	0	0	1
Total	17	126	50
Median	1	3	3

BMRTC is not a sarcomatous variant of Wilms' tumour. Clinically, in contrast to Wilms' tumour, BMRTC shows a marked male preponderance and a higher rate of bone metastasis (60% compared to 1% in Wilms'). In our experience of 50 cases of BMRTC, unlike Wilms' tumour these are rare in children under the age of 1 year (Marsden and Lawler 1980 and also see Table 1). Histologically BMRTC consists of polygonal cells with a highly vascular framework and the absence of blastemal cells and metanephric differentiation. Electron microscopic studies have suggested common features between BMRTC and mesangial cells and immunological studies support the view that this tumour is not a variant of Wilms' but a distinct separate entity (Marsden et al. 1978 and Kumar et al. 1980). The results of the present study provide additional evidence that the two tumour types behave differently in vitro (Table 2).

Applications of tissue culture techniques have in the past been most helpful in aiding the histological diagnosis of childhood neoplasms, in particular neural tumours. For instance, even in the absence of raised urinary catecholamines and a doubtful histological picture, a tumour can be diagnosed as a neuroblastoma by its behaviour in vitro. In the present paper we describe characteristic features of childhood kidney tumours (Wilms', mesoblastic nephroma and BMRTC) in vitro. Growth patterns of BMRTC, mesoblastic nephroma and Wilms' tumour are distinct and that of BMRTC can be profoundly altered by the addition of fibronectin to tissue culture medium.

Table 2. Some distinguishing features of childrens' kidney tumours

Tumour type	Histology[a]	Age at diagnosis[a]	Sex Preponderance[a]	Site of metastases[a]	In vitro studies				
					Morphology	Staining with Wilms' antiserum[b]	Fibronectin	Migration in collagen	Effect of the addition of fibronectin on migration in collagen
Mesoblastic nephroma	Fibroblastic spindle cells	Median 1 year (none above 2 years old)	None	Rare	Spindle shaped shaped cells. No nuclear polymorphism	+	+	−	−
Wilms'	Mixed: epithelial and blastemal cells	Median 3 years (16% under 1 year)	None	Mainly Lung; Bone: 1%	Mainly polygonal & epithelial like; a few fibroblastic. Nuclear polymorphism marked	+	+	−	−
BMRTC	Polygonal cells with prominent capillary pattern	Median 3 years (Rare under 1 year)	Male 8 times	Mainly bone: 60%	Polygonal cells with marked contact inhibition	−	−	+	+ (profoundly reduced)

[a] see Marsden et al. (1984)
[b] see Kumar et al. (1980)

Materials and methods

Details for processing biopsy material and tissue culture methods can be found in our previous publication (Waghe et al. 1971).

A. Tumours. Tissues (2 Wilms' tumours, 2 mesoblastic nephromas, 2 BMRTC, 2 fetal kidneys and 2 normal kidneys) were fresh biopsies of fragments stored in liquid nitrogen in a freezing medium containing medium 199 (6 parts), heat inactivated fetal calf serum (3 parts) and dimethyl sulphoxide (1 part). Macroscopically healthy looking tissue fragments (4–5 mm^3) were minced using a pair of curved scissors. The cell suspension was vigorously pipetted up and down a pipette to make, as far as possible, a single cell suspension. Kidney tumours are normally very friable and a cell suspension can easily be produced without enzyme treatment. The cell suspension was allowed to stand for 5 min to allow large fragments to settle. The supernatant was removed and washed twice with a large volume of complete medium (medium 199 containing 15% heat inactivated fetal calf serum with penicillin and streptomycin). The cell pellet was resuspended in fresh medium and transferred to Falcon tissue culture flasks (T75). Two days later the medium was carefully renewed and thereafter every 72 h. The cells were usually semiconfluent within 10–14 days. Cells were subcultured using 0.05% trypsin (Difco). All tissue culture studies were done using only primary cultures.

B. Collagen gels. Collagen type I was prepared from rat tail tendons as previously described by Ehrman and Gey (1956) and Elsdale and Bard (1972). Rat tail tendons were extracted in 0.5 M acetic acid, and dialysed for 2–3 days against distilled water to remove acetic acid. The dialysed solution of tropocollagen was then centrifuged at $10{,}000 \times g$ overnight at 4° C. Gels of native collagen were prepared by adding 1 ml of X10 concentrated medium (without serum) and 0.5 ml of 4.4% sodium bicarbonate to 8.5 ml of collagen solution. The mixture was kept on ice and rapidly mixed and dispensed into 35 mm^2 Falcon tissue culture petri dishes. Within a few minutes the gels had set and were ready to use or were stored in a humidified atmosphere with or without an overlay of medium. The gels thus formed consist of a lattice of collagen films with a banding pattern similar to collagen fibres in situ. For further details the reader is referred to the original papers of Ehrman and Gey (1956); Elsdale and Bard (1972), and those of Schor and co-workers which have been reviewed by Yang and Nandi (1983). It is relevant to point out that the gels of native collagen are entirely different from heat or ammonia-denatured collagen.

Fibronectin. Human fibronectin was a gift from Dr. R. Brown and antiserum to human fibronectin prepared in rabbits was generously provided by Dr. P.L. Walton.

Fibronectin-depleted serum was prepared by passing fetal calf serum through a gelatin-sepharose column and washing the column with serum-free tissue culture medium containing antibiotics. The serum was centrifuged at $10{,}000 \times g$ overnight at 4° C. The supernatant was aliquoted and stored at −20° C. This serum was used to prepare fibronectin-depleted complete medium (FDCM), the final concentration of serum being 15%.

Tissue culture technique. 2 ml of cell suspension (5×10^4) were plated into 35 mm^2 tissue culture dishes with or without collagen gel. The following day, the dishes were washed with phosphate-buffered saline (PBS) and 2 ml of fresh medium was added. Thereafter the medium was changed every 72 h.

To examine the effect of fibronectin, cell suspensions were resuspended in FDCM and inoculated into petri dishes containing collagen gel. The following day the medium was changed with FDCM containing 0, 1, 5 or 25 μg fibronectin in 1 ml of medium.

The cells were examined using a Leitz inverted phase contrast microscope. The number of cells that were growing on top of the collagen and those that had migrated into the collagen gel were assessed (Schor et al. 1981a, b). For this an eyepiece graticule was used and at least 10 different fields were scored in each petri dish. Cells on collagen were generally flat and well spread and those that had migrated into the gel were spiky and irregular in morphology. Clearly, cells penetrated to different depths in the collagen and no attempt was made to determine the actual distances travelled within the collagen. The procedure of counting

cells was repeated daily for up to 10 days. Prior to terminating an experiment tissue culture dishes were twice washed with PBS and the collagen gel was dissolved using a 0.05% collagenase for 4 h at 37° C. Any adherent cells were removed using 0.05% trypsin. The cell numbers were determined using a Coulter counter.

Immunofluorescence procedure. The indirect immunofluorescence technique was used to examine the staining reaction of tissue cultured cells. For this, cell suspensions were grown in FDCM in Leighton tubes containing glass coverslips. The coverslip cultures were twice washed with PBS, fixed in cold acetone for 10 min and treated with fibronectin antiserum (diluted 1:1000 in PBS) for 20 min at room temperature. The controls were treated with an equivalent dilution of normal rabbit serum. Coverslips were washed in PBS for 30 min in a tray which was gently rocked by placing it on top of a slightly unbalanced centrifuge. The washed coverslips were treated with fluorescein isothiocyanate labelled goat anti-rabbit serum (1:40 dilution, Wellcome Laboratories, Beckenham, Kent) for 20 min at room temperature. The cells were washed in PBS, mounted in glycerol-saline (1:1) and examined under a Leitz incident light fluorescence microscope using appropriate UV filters. All slides were read blind and photomicrographs were taken using high speed Kodak films (27 DIN).

Results

Histology and growth characteristics

Two Wilms' tumours containing well differentiated tubules were studied (Fig. 1a). The appearance of these tumours in tissue culture was fairly characteristic. In addition to macrophages, two distinct types of cell were seen. The majority were polygonal and epithelial-like, either isolated or in sheets and the other type was fibroblastic (Fig. 1b). The frequent nuclear pleomorphism suggested that most of the cells were neoplastic.

Histologically both mesoblastic nephromas consisted of fibroblastic spindle cells in which embedded renal tubules and glomeruli were seen (Fig. 2a). The tumour cells were not as hyperchromatic as in undifferentiated nephroblastoma. In culture mesoblastic nephroma consisted of uniformly dispersed spindle-shaped cells containing one to three nucleoli (Fig. 2b). Polymorphic and bizarre shaped nuclei were rare. Beside fusiform cells, a few polygonal cells were also present.

Two BMRTC had a rather characteristic morphology, the predominant component consisting of polygonal cells with a prominent capillary pattern (Fig. 3a). The cultures of both BMRTC showed marked contact inhibition. The vast majority of cells were polygonal in shape (Fig. 3b). Normal kidney cultures from 2 children of 3 and 5 years of age and 2 embryonic kidneys (16 and 17 weeks of gestation) grew as an orderly monolayer of polygonal epithelial type cell (Fig. 4).

Growth on collagen

Normal, fetal kidney and tumour (Wilms', BMRTC and mesoblastic nephroma) derived cells grew equally well on collagen and their pattern of growth was essentially similar to that described earlier. The typical morphology of cells is shown in Fig. 5. As regards their growth into collagen gel, significant differences were found.

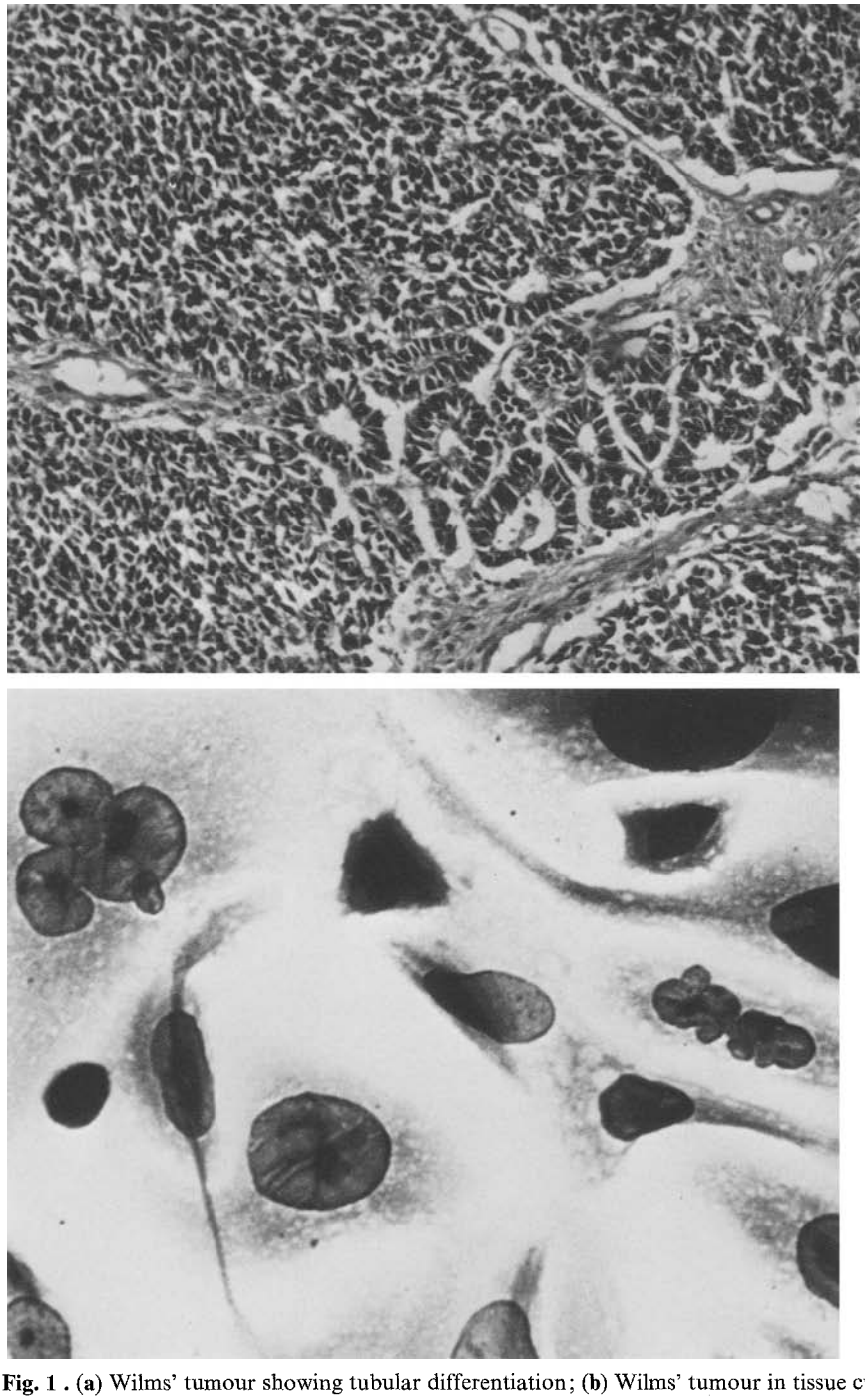


Fig. 1 . (a) Wilms' tumour showing tubular differentiation; **(b)** Wilms' tumour in tissue culture

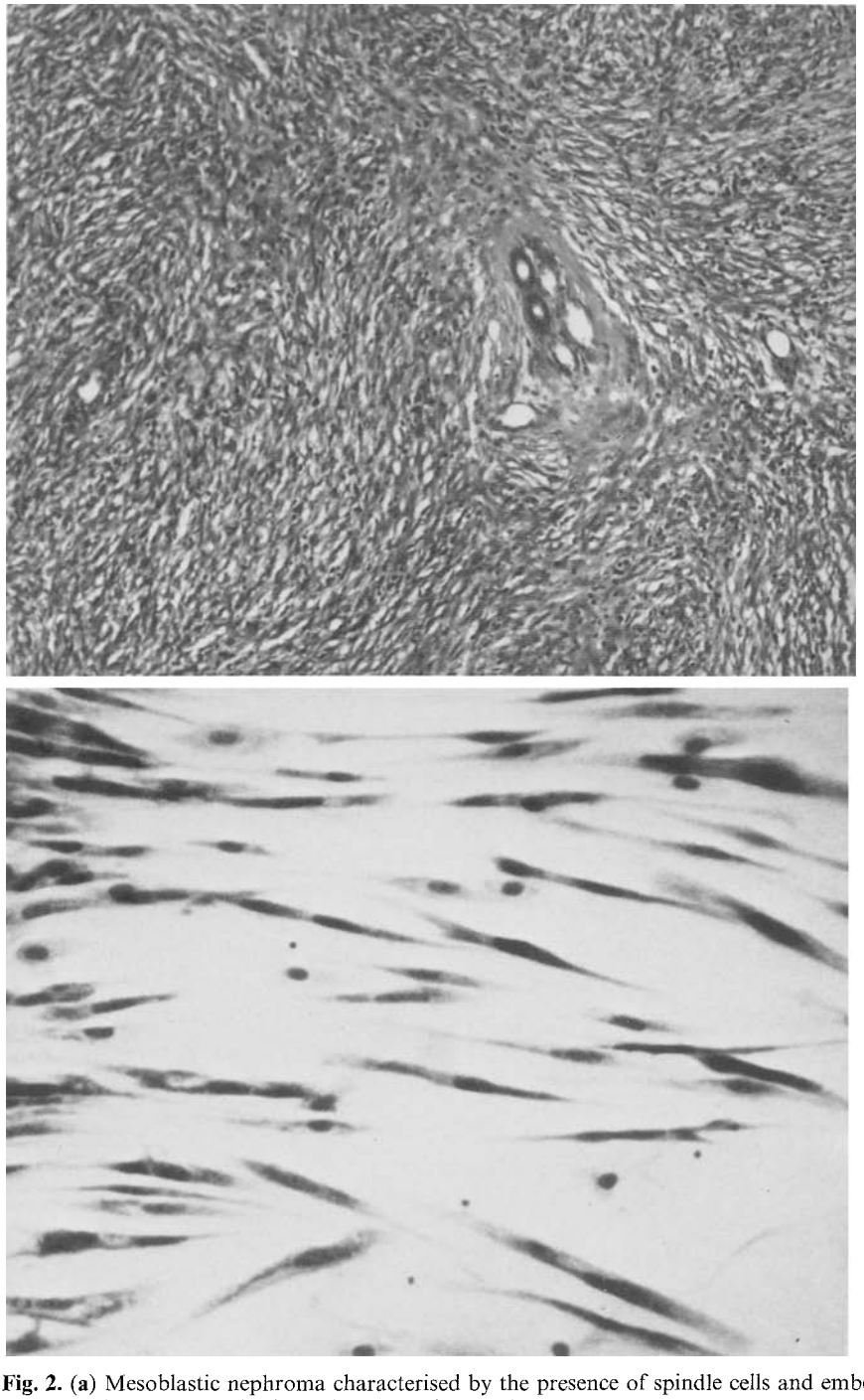


Fig. 2. (**a**) Mesoblastic nephroma characterised by the presence of spindle cells and embedded renal tubules. (**b**) Mesoblastic nephroma in tissue culture

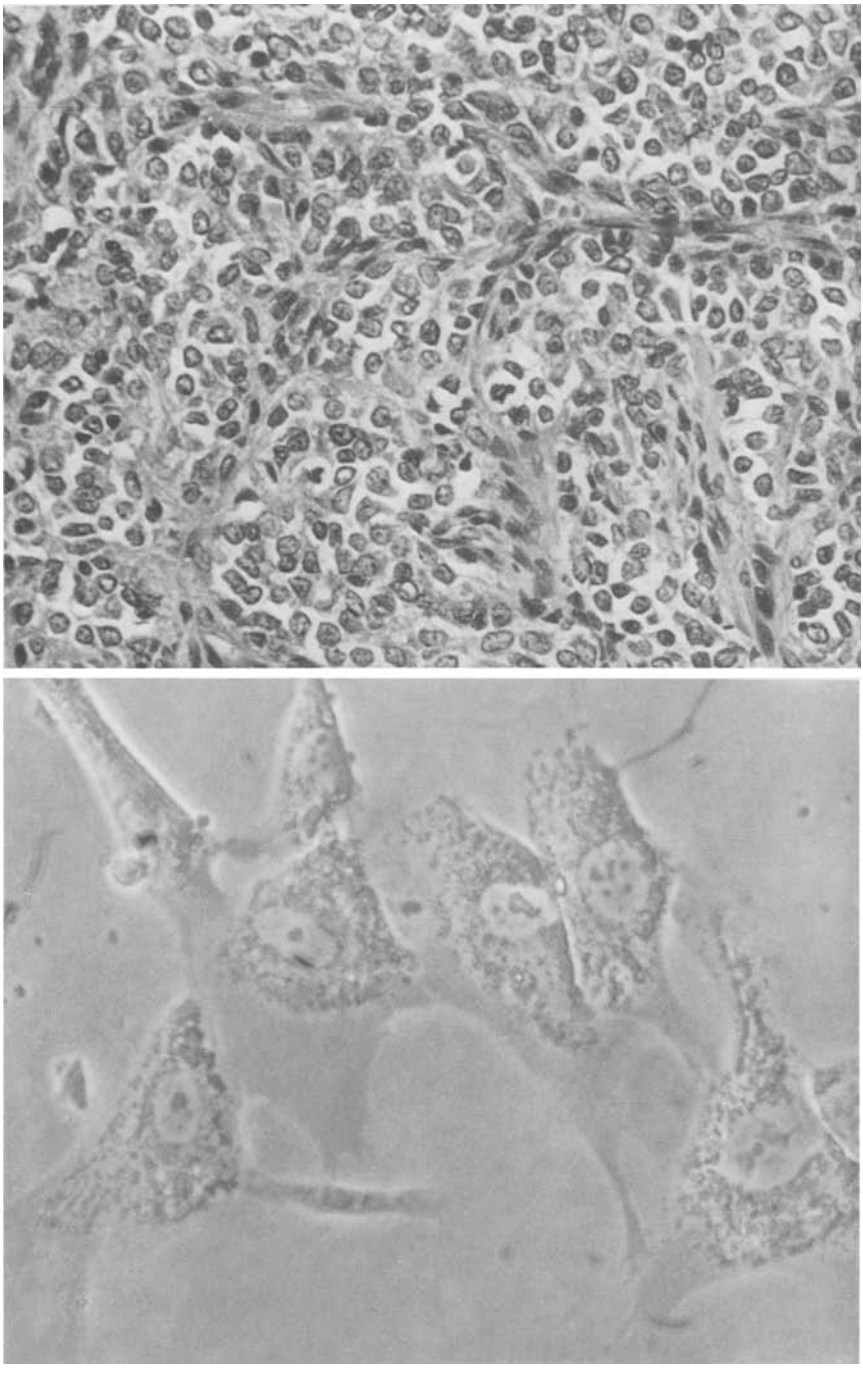


Fig. 3. (**a**) Bone metastasizing renal tumor of childhood (BMRTC). Note characteristic vascular framework and absence of blastoma. (**b**) BMRTC in tissue culture

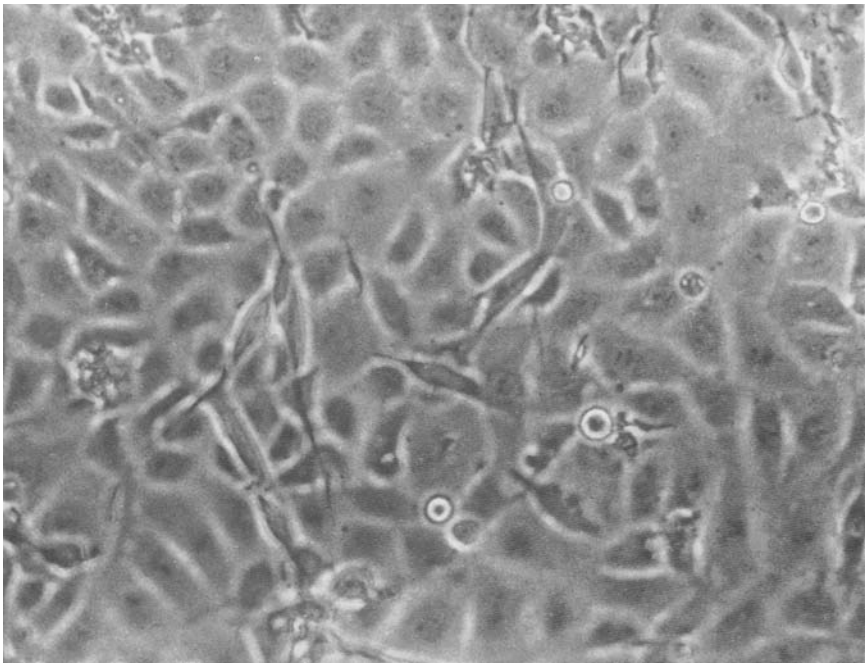


Fig. 4. Normal kidney in tissue culture

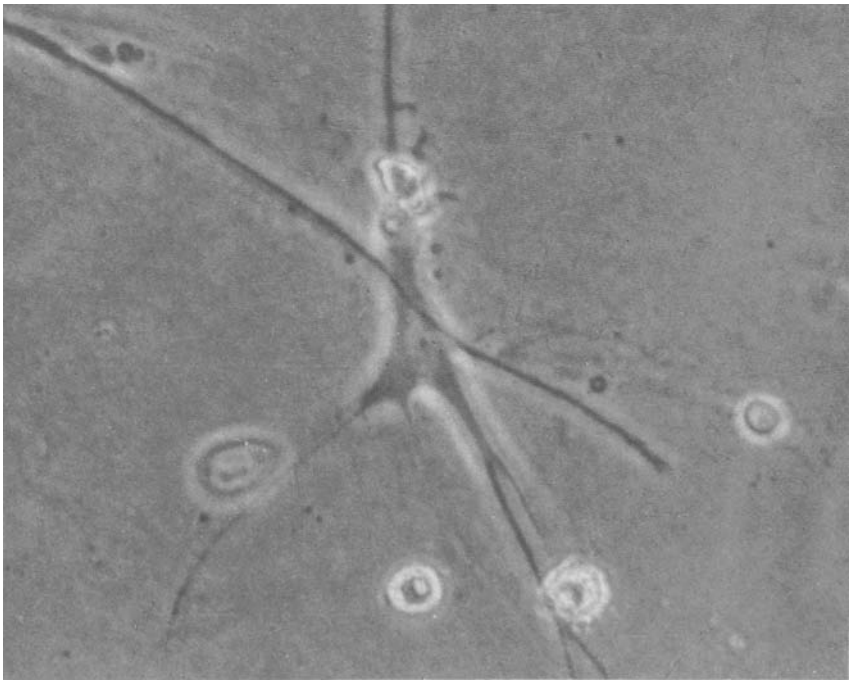


Fig. 5. Migration of BMRTC into collagen – cells migrate to various depths thus one cell is in focus, five others are not.

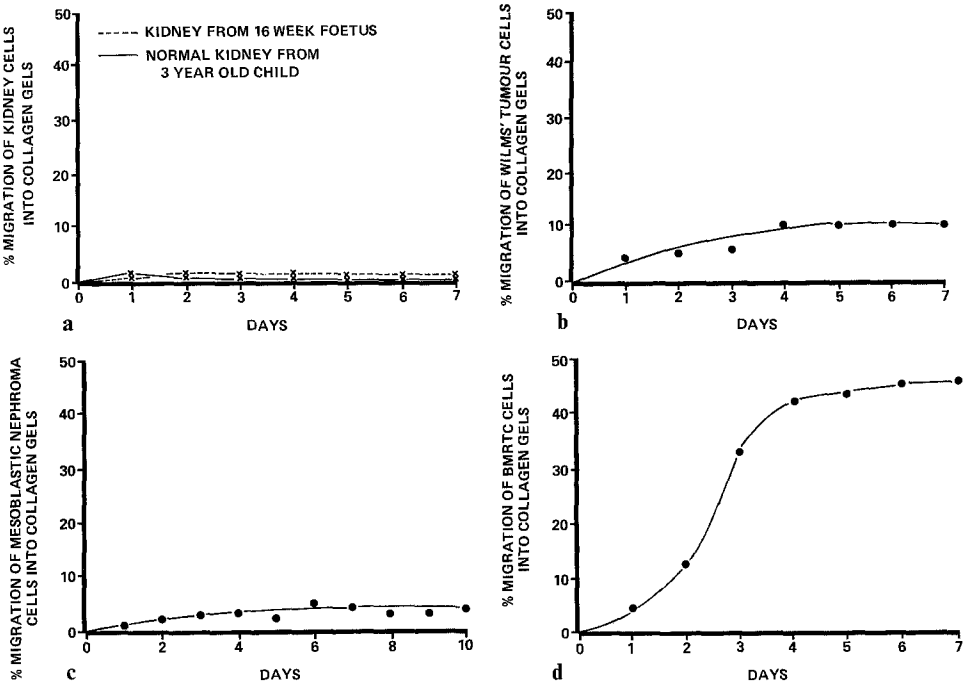


Fig. 6. Quantitation of cell migration into collagen: (**a**) Normal and fetal kidney; (**b**) Wilms' tumour; (**c**) Mesoblastic nephroma; (**d**) BMRTC – in contrast to (**a**), (**b**) and (**c**) note marked migration

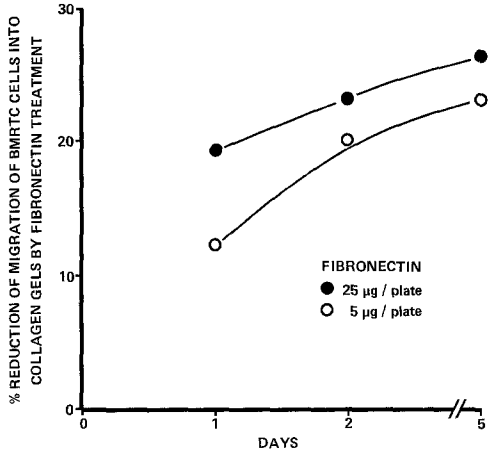


Fig. 7. Inhibitory effect of fibronectin on migrations of BMRTC into collagen

Normal and fetal kidney cells failed to migrate into collagen even after a period of 7 days. In fact fewer than 2% of cells migrated into the collagen (Fig. 6a). The percentage of Wilms' tumour and mesoblastic nephroma derived cells that moved into the collagen was a little higher and gradually increased over a period of 7 days, but failed to reach 10% (Fig. 6b and

c). On the other hand, tumour cells from both BMRTC showed a marked tendency to invade collagen gels. By day 7 approximately 50% of cells had entered the gel matrix (Fig. 6d).

The addition of fibronectin to tissue culture medium significantly reduced the migration of BMRTC into collagen (Fig. 7). When the experiment was terminated the total number of cells in both treated and control dishes was the same (data not shown). Thus the addition of fibronectin had no effect on cell proliferation. The addition of fibronectin to either Wilms' tumour, kidney or mesoblastic nephroma culture had no effect on cell migration (data not shown).

Immunofluorescence staining. Tissue cultured cells of normal kidney, fetal kidney, Wilms' tumour and mesoblastic nephroma stained very intensely with fibronectin antiserum. The pattern of staining was not uniform and varied from cell to cell. No particular pattern of staining was prevalent in any of the above 4 sources of tissues. Figure 8 shows the major types of immunofluorescence seen. The first was fibrillar either in short strands or forming a network, especially at the periphery of a cell (Fig. 8a). Some of the fibrils ran from one cell to another. In the second type most of the cell surface was devoid of staining except for irregular shaped amorphous lumps of fibronectin. Usually a large amorphous network of staining material was present in the intercellular spaces in among these cells. The third type of staining was the most frequent and was a mixture of the two staining patterns, i.e. fibrillar and amorphous (Fig. 8c).

Discussion

That the characteristics of cells in tissue culture can be valuable in distinguishing the tumours described is apparent from the results of this study. The technique was helpful in distinguishing Wilms' tumour from cellular mesoblastic nephroma. Similarly the pattern of growth of BMRTC was clearly distinct from Wilms' tumour with which it has been confused. The present observations support previous reports that BMRTC should be regarded as a distinct and separate histological entity (see Table 1 and 2). The ages of children at diagnosis with Wilms' tumour and BMRTC were similar with the exception that BMRTC was relatively uncommon under the age of 1 year. On the other hand all 17 mesoblastic nephromas occurred in children under the age of 22 months (Table 2). At present it is difficult to be certain about the nature and origin of these tumours. The following is a proposed diagrammatic representation of histogenesis of children's primary renal tumours.

Clinically a marked difference between Wilms' tumour is that the latter frequently metastasise to bone. In contrast, Wilms' tumour is often metastatic in lung, while bone metastasis is rare. Nicolson (1982) in a review of cancer metastasis based on nearly 800 publications wrote "surprisingly few comprehensive studies on the process of metastasis in man or in experimental animal models have been undertaken, although metastasis is one of

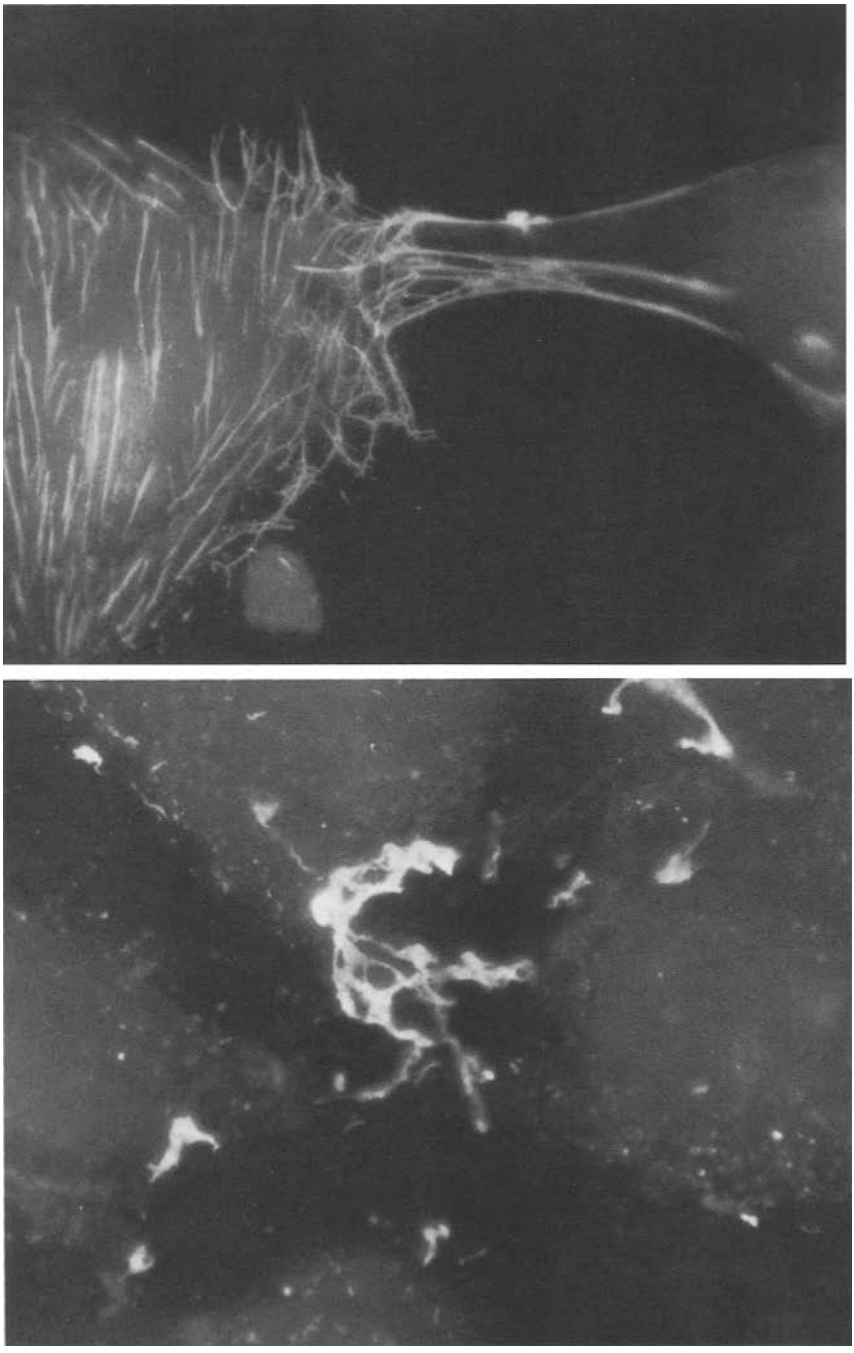


Fig. 8. Distribution of fibronectin in tissue cultured cells. Immunofluorescence staining revealed fibronectin was present in 3 main patterns: (**a**) Fibrillar; (**b**) Amorphous lumps; (**c**) "mixture" of (**a**) and (**b**)

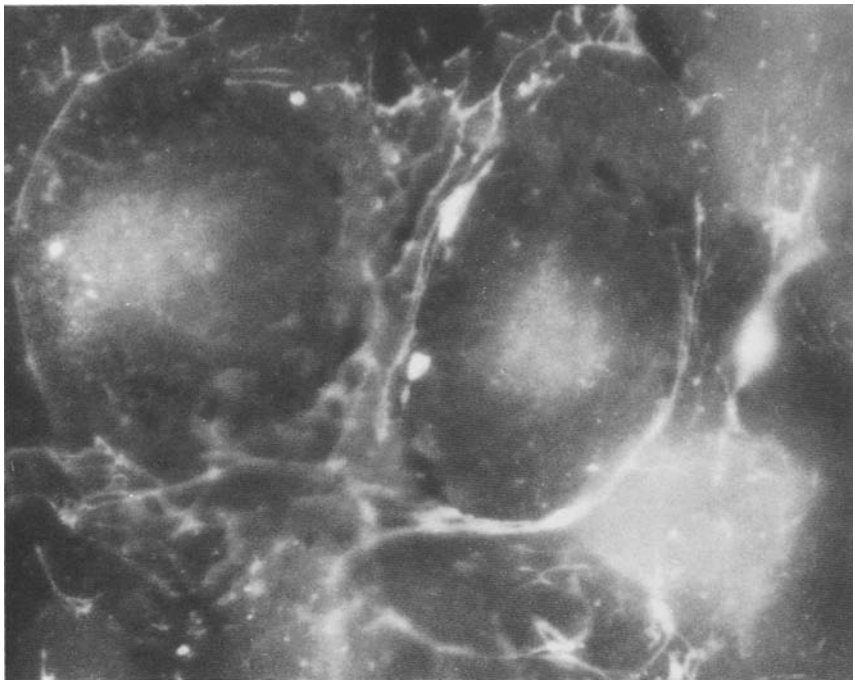
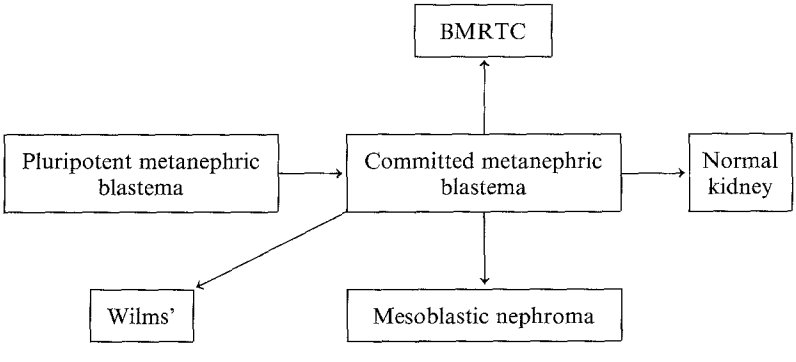


Fig. 8c



the most important events in the pathogenesis of cancer and accounts for most human cancer mortality". He went on to state that metastasis is a complex cascade of events wherein malignant cells invade normal tissues, enter circulation, detach and travel to other parts of the body where they stop, become vascularized, invade and multiply to form a metastatic mass. Why one tumour type prefers one organ and not another for metastasis is not known. Nicolson (1982) has discussed the two classical views: one of Ewing (1928) that metastasis occurs by 'mechanical factors' which are due to the anatomic structure of the vascular system and Paget's (1889) 'seed and soil' hypothesis. In addition he has given an account of much

of the other relevant literature. The endocrine status of a host is thought to play an important role in tumour metastasis. In the case of Wilms' tumour and BMRTC it is not known whether hormones have an influence on metastasis. For some types of cancer the organ preference for distant metastasis is so predictable that certain tumours can be cured by surgical excision of the target organ before malignant colonisation as has been shown in a murine model (Kotzin and Strober 1980).

Collagen is an important component of bone and for this reason was used for tumour culture studies. Many types of collagens have been described. Bone contains mainly type I. Denatured collagen has in the past been frequently used for the tissue culture of fastidious cells. A number of papers expecially those of Schor and co-workers have shown marked differences as regards cell attachment, proliferation and migration of cells on native and denatured collagen (Schor et al. 1983; Yang and Nandi 1983). Collagen in vivo exists in a native form, therefore an in vitro study is more meaningful if native collagen is used. The results of the present study show that BMRTC cells can migrate into type I native collagen. On the other hand normal and fetal kidney, and Wilms' tumour derived cells although they proliferate well on collagen gels, do not penetrate it. The mechanism by which collagen affects cell behaviour is far from known but Grobstein in 1953 and many others have suggested that the cell substratum plays an important role in cell proliferation and morphogenesis (see Yang and Nandi 1983). Collagen itself may have an influence on cell morphology.

As regards the migration of BMRTC into collagen the role of proteolytic enzymes is not known. Marked differences in the levels of degradative enzymes among normal, benign and malignant tissues have been recorded. Generally, malignant cells have increased levels of proteolytic enzymes (reviewed by Nicolson 1982). Our scanning EM photomicrographs show that the cells of BMRTC migrated into collagen without apparent disruption or degradation of collagen fibres – an observation similar to one seen in many other types of cells (Fig. 9). Schor et al. (1980a) using Chinese hamster ovary and melanoma cells concluded that both cell types migrated into collagen without solubilizing radio-labelled collagen. It is possible that a physical phenomenon is involved in the migration of BMRTC. Carter (1965 and 1967) studied cell motility on palladium coated surfaces. He was able to control cell movement by progressively increasing metal deposition on a surface of cellulose acetate. This provided a gradient for cell to substratum adhesion and cells were found to move up this adhesion gradient in a highly directional manner. The phenomenon was called 'haptotaxis'.

Fibronectin is an important component of the extra-cellular matrix. At least two forms (plasma and cellular) have been recognised. Among the functions of fibronectin are its role in opsonization, adhesion of cells to substrata and in cell spreading and morphology. Malignant cells normally have either no, or low levels, of fibronectin compared to normal tissues. The distribution of fibronectin generally is in a fibrillar matrix around the cells, but the characteristic pattern of fibrils varies with the cell type and culture conditions (for reviews see Yamada 1981 and Hynes 1981).

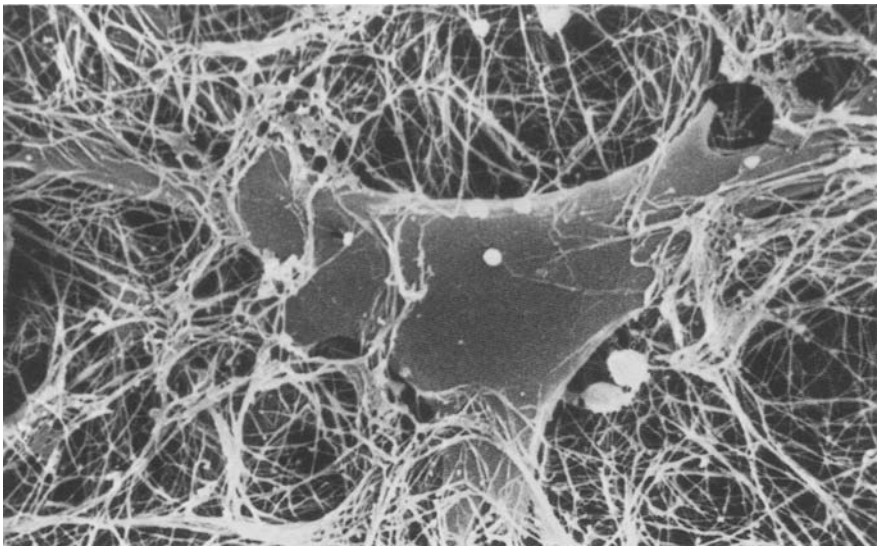


Fig. 9. Scanning EM photomicrographs of tissue cultured cells on collagen – the presence of apparently intact collagen fibres on and around a cell can be seen (technical help of Ms. A. Erroi with Scanning EM is very gratefully acknowledged)

In the present study no one staining pattern was particularly marked in any culture of the various tissues studied. Fibrillar, amorphous and a mixture of these two types were found in normal kidney, fetal kidney, Wilms' tumour and mesoblastic nephroma in marked contrast to BMRTC. Hynes (1981) has stated in his review that the staining pattern can vary as the cells begin to spread. For instance, 2 h after plating cells tend to have a punctate pattern of staining frequently arranged in a circle, whereas after 5 h the same cells show fibronectin arranged as fibrils. Hynes has cautioned that if immunofluorescence is the only procedure used to detect fibronectin, doubt can remain as to its origin since serum fibronectin can bind to the cell and form a fibrillar network. In the present study, in order to exclude the possibility that the immunofluorescence was due to serum used in the tissue-cultured medium, cells were grown in fibronectin-depleted medium. The pattern of staining was not affected by the use of fibronectin-depleted serum.

The readdition of fibronectin can influence the migratory behaviour of cells. In some types of cell, migration is enhanced and in others retarded (Schor et al. 1983). Ali et al. (1977) have observed that the addition of fibronectin to transformed cells which lack it, also causes changes in the organisation of microfilaments inside the cell. The electron microscopic study of Singer (1979) showed that extracellular fibronectin is colinear with intracellular microfilament bundles.

We have found that BMRTC cells lack fibronectin and the addition of fibronectin markedly reduced the migration of these cells into collagen. Whether this observation represents the in vivo situation is not known i.e.

if fibronectin can influence the metastatic ability in BMRTC bearing children is not certain. If it does mimic the in vivo situation then there is a paradox, as normal human serum contains a large quantity (0.3 mg/ml) of fibronectin – a concentration far higher than the one used in the present in vitro studies. However, Brown (1983) has pointed out a major deficiency of the assays employed to estimate fibronectin. He argues that fibronectin can be easily degraded to give fragments which may have altered reactivity in an assay and also impaired function. Therefore it is important to measure only functional fibronectin. Future studies need to ask the following questions to resolve the relevance of fibronectin in childhood kidney tumours:

1. Do BMRTC cells inherently lack fibronectin?
2. Do BMRTC cells possess degradative enzymes which make them lose receptors for serum fibronectin?
3. What effect does the readdition of fibronectin have on the cytoskeleton of BMRTC?
4. What is the level of functional fibronectin in the sera of children with BMRTC?

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