Journal of Cell Science 108, 2905-2916 (1995)
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Metastasis-associated 5T4 oncofoetal antigen is concentrated at microvillus projections of the plasma membrane

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SUMMARY

The 5T4 oncofoetal antigen is a 72 kDa glycoprotein defined by a monoclonal antibody raised against human placental trophoblast and is expressed in many different carcinomas but detected only at low levels in some normal epithelia. Immunohistochemical analysis of the patterns of expression in colorectal carcinomas has indicated a significant association between the presence of the antigen in tumour cells and metastatic spread. A cDNA encoding the 5T4 molecules has been isolated and the extracellular portion contains several leucine-rich repeats which have been implicated in cellular interactions. To study the cell biological role of 5T4 molecules, murine L cells (A9 derivative) were stably transfected with 5T4 cDNA under the control of the CMV immediate-early promoter. The 5T4-expressing cells exhibited a more spindle-shaped morphology compared to the vector alone transfected cells. Confocal immunofluorescence microscopy revealed a 'polkadot' pattern of 5T4 antigen expression, heterogeneous in intensity between cells, but distributed over the entire cell surface. Transmission and scanning electron microscopy showed that the 5T4 antigen is concentrated at microvillus projections of the plasma membrane both in the transfected A9 cells and in various carcinoma cell lines. Such projections express an array of surface molecules which function in cell adhesion and motility. This association of 5T4 antigen with microvillus projections was also observed in various carcinoma cell lines. 5T4 expression in A9 cells was also associated with an altered pattern of cell division, decreased cell-substratum adhesion and increased cellular motility. These results support the hypothesis that 5T4 molecules may have a direct function in trophoblast and tumour cell invasion processes.

Key words: 5T4, metastasis, microvillus

INTRODUCTION

Comparisons between the growth and invasive properties of embryonic trophoblast and tumour cells have stimulated the search for functional overlaps in cell surface molecules. The 5T4 oncofetal antigen is a 72 kDa glycoprotein defined by a monoclonal antibody (mAb 5T4) raised against wheat germ agglutinin isolated glycoproteins from human placental syncytiotrophoblast microvillus membrane (Hole and Stern, 1988). The antigen is expressed by trophoblast throughout gestation but shows a restricted expression in other pregnancy-associated or adult tissues with only weak expression on some specialised epithelia. By contrast it is expressed by many human carcinomas and a variety of transformed embryonic and carcinoma-derived cell lines (Southall et al., 1990; Hole and Stern, 1988). Recent studies have demonstrated a significant correlation between 5T4 antigen expression by malignant cells of colorectal and gastric (Starzynska et al., 1992, 1994), and ovarian (Wrigley et al., 1994) tumour tissue, and metastasis and disease progression. The correlation between 5T4 antigen expression in tumour cells and stage of cancer suggests that the appearance of this molecule reflects a change which may contribute to the process of metastatis.

The 5T4 antigen has been biochemically characterised as a 72 kDa glycoprotein, consisting of a 42 kDa core protein with extensive N-linked glycosylation (Hole and Stern, 1990). A cDNA encoding the 5T4 oncotrophoblast glycoprotein has a deduced amino acid sequence, indicating the mature protein to be membrane bound comprising a 310 amino acid extracellular region with seven N-linked glycosylation sites and a short 44 amino acid cytoplasmic tail (Myers et al., 1994). No specific homologies have been identified at the DNA level but comparison with protein databases has revealed the presence of seven leucine-rich repeats (LRRs) in the extracellular portion.

LRRs are short sequence motifs present in a range of proteins with diverse functions and tissue distributions (reviewed by Kobe and Deisenhofer, 1994). LRRs are believed to mediate specific protein-protein interactions. Proteins with adhesion receptor functions represent the largest group in the LRR superfamily, although it is not clear whether the LRRs are the functional entity in all molecules. Several LRR-containing proteins with adhesive properties are found in *Drosophila*, including toll (Keith and Gay, 1990), connectin (Nose et al., 1992; Gould and White, 1992) and chaoptin (Reinke et al., 1988), which orient cells during development. Mammalian LRR-containing proteins with possible adhesive functions include the proteo-

glycans decorin, biglycan and fibromodulin, which are involved in cell attachment and interact with collagens (Fisher et al., 1989; Kresse et al., 1993) and platelet membrane glycoprotein 1b-1X-V, which is a receptor for von Willebrand factor, a molecule required for the adhesion of platelets to exposed subendothelium at sites of vascular damage (Hickey et al., 1989, 1993). Since 5T4 molecules contain LRRs, it is possible that the antigen may facilitate trophoblast and tumour cell invasion through modulation of cell adhesion. In addition, the N-linked carbohydrate of 5T4 molecules might also confer functional interactive properties.

Studies of the cellular location of molecules can often give an insight into their functions. Cell adhesion molecules are often concentrated at focal contact points, specialised membrane domains where actin-filament bundles terminate at sites of attachment to the extracellular matrix (Pavalko and Otey, 1994). Examples include some integrins (Burridge et al., 1992; Juliano and Haskill, 1993) and syndecan-4 (Woods and Couchman, 1994). Other molecules are concentrated at microvillus projections and blebs of the plasma membrane. Examples include ICAM-1 (Carpen et al., 1992), some N-CAM molecules (Speirs et al., 1993), the vitronectin receptor $\alpha5\beta3$ (Lakkakorpi et al., 1993), L-selectin (Erlandsen et al., 1993), $\alpha4\beta7$ (Berlin et al., 1995), ELAM-1 (Tomczok et al., 1994) and collagenase (Moll et al., 1990). The functional significance of microvillus projections and blebs is poorly understood, but the array of molecules found on such projections suggests a role in cellular interactions. Rovensky et al. (1992) reported that immortalised or transformed tumorigenic fibroblasts exhibited an increased number of microvillus projections compared with normal cells, and suggested that such projections may serve as a morphological marker of neoplastic transformation. In contrast to molecules with a distinctive plasma membrane distribution, some molecules are diffusely dispersed, including the integrins $\alpha2\beta1$ and $\alpha3\beta1$ (Klein et al., 1990; Elices et al., 1991), LFA-1 (Berlin et al., 1995) and Mac-1 (Erlandsen et al., 1993).

Several molecules involved in cellular interactions associate with the cellular cytoskeleton. For example, some integrins and cadherins associate with actin filaments, via complexes of cytoplasmic proteins including α-actinin, talin, the catenins and radixin (Pavalko and Otey, 1994). Such associations serve to link extracellular signals with fundamental processes in cells such as shape change, adhesion and motility.

To study the cell biological role of 5T4 molecules, murine L cells (A9 derivative) were stably transfected with 5T4 cDNA under the control of the CMV immediate-early promoter. The cellular location of 5T4 expression in the 5T4-transfected cells, as well as in various human carcinoma cell lines which naturally express the 5T4 antigen, was studied by immunolabelling using confocal immunofluorescence microscopy, and transmission and scanning electron microscopy. The morphology, pattern of cell division, cell-substratum adhesion, cell-cell aggregation, cellular motility and invasiveness of 5T4-transfected and vector alone control-transfected A9 cells were compared.

MATERIALS AND METHODS

Transfection of the L cell line with 5T4 cDNA

Cultures of the murine A9 cell line (a derivative of the L cell line, a gift from Dr J. Boyle, Paterson Institute for Cancer Research) were transfected with 5T4 cDNA as previously described (Myers et al., 1994). Briefly, 5T4 cDNA was inserted into the mammalian expression vector $pCMV\alpha_{neo}$ and this construct was transfected into the A9 cell line, using Transfectam (Promega) and serum-free Optimem medium (Gibco BRL). Foetal calf serum (5%, FCS, Gibco BRL) was added 24 hours later, and neomycin-resistant clones were selected by addition of G418 (1 mg/ml, Gibco BRL) after a further 24 hours. Two independent clones of both 5T4- (A9-5T4a and A9-5T4b) and vector alone- (A9-H12 and A9-D4) transfected cells were isolated. The cells were routinely cultured in MEM alpha medium (MEMα, Gibco BRL) containing 0.25% FCS and 0.5 mg/ml G418. For assays, cells were either cultured in serum-free MEMα containing 10 μg/ml transferrin (Collaborative Research), or in MEMα containing 0.25% or 5% FCS.

Cell culture

The EJ cell line (human bladder carcinoma), was cultured in RPMI medium (Gibco BRL) containing 5% FCS. The HT29 (human colon carcinoma) and the Balb/c 3T3 (murine fibroblast) cell lines were cultured in DMEM medium (ICN Flow) with 5% FCS. The Jeg3 and JAR cell lines (human choriocarcinomas) were cultured in MEMα medium with 10% FCS. All cell lines used are tested regularly for mycoplasma contamination, with negative results each time.

Southern blot analysis

High molecular mass DNA was prepared from transfectant clones by lysis of cultured cells in buffer containing 75 mM NaCl, 25 mM EDTA, 1% SDS and 200 μg/ml proteinase K followed by phenol/chloroform extraction and ethanol precipitation. In order to determine copy number this DNA was digested with *Eco*RI and electrophoresed along with dilutions of *Eco*RI-digested 5T4 cDNA-pCMVαneo construct and blotted to Biotrans nylon membranes (ICN) as described by Southern (1975). The filters were then prehybridized at 65°C in phosphate buffer containing 1% BSA and 7% SDS (Church and Gilbert, 1984) for at least 1 hour. Hybridization was carried out in the same buffer overnight at 65°C with ^{32}P-labelled 5T4 cDNA prepared by random priming (Feinberg and Vogelstein, 1984). Filters were washed 2-3 times for 10-15 minutes with 20 mM sodium phosphate, 100 mM NaCl, 1% SDS at 65°C and exposed to Kodak X-Omat AR film at −70°C.

Western blot analysis

For western blot analysis, A9-H12, A9-D4, A9-5T4a, A9-5T4b, EJ, HT29 and JEG3 cell lines were lysed in 25 mM Tris-HCl-buffered saline, pH 7.4 (TBS), containing 0.5% NP40 at 10^7 cells/ml. Aliquots of 10 μl (10^5 cells) cell lysates were separated by SDS-polyacrylamide gel electrophoresis using 10% (w/v) acrylamide and 0.1% SDS (Laemmli, 1970). Proteins were transferred to nitrocellulose using a Multiphor Novoblot electrophoresis transfer system. The 5T4 antigen was labelled with mAb 5T4 and detected by ECL (Amersham). The resulting bands were analysed using UVB Gel Base Densitometry Software.

Cell proliferation

For assessment of cell proliferation, 1×10^4 cells were seeded into wells of 6 mm × 35 mm well plates, in 2 ml of serum-free MEMα containing 10 μg/ml transferrin. After 24 hours, the cultures were washed twice in PBS, to remove unattached cells, and fresh medium, either serum-free or containing 0.25% or 5% FCS, added. The cell number was determined (duplicate wells) following trypsinisation at this time (day 1) and up to 16 days thereafter. Fresh medium was added twice during this time.

Phase-contrast and time-lapse video microscopy

The transfected A9 cells (clones A9-H12, -D4, -5T4a and 5T4b), cultured in MEMα with 0.25% FCS, were visualised and photographed using an Olympus CK2 phase-contrast microscope.

The behaviour of A9-5T4a cells was compared with control A9-H12 cells using a time-lapse video microscopic system established by Allen (1987). Cells were cultured in 25 cm^2-base flasks, in MEMα containing 0.25% FCS, and maintained at 37°C in a hot box mounted on a Leitz Diavert microscope. Time-lapse video was performed over a period of 15 hours, using an animation control unit (EOS Ltd, Barry, Wales, UK) and Umatic SP format edit tape recorder. Two frames were exposed every 20 seconds, leading to a 250× speed increase when replayed at normal rates (25 frames/second).

Immunofluorescence microscopy

For immunofluorescence, cells were cultured on 13 mm glass coverslips (no. 0, Chance Proper Ltd), in MEMα containing 0.25% FCS. All procedures were carried out at room temperature unless otherwise indicated. PBS containing 1% (w/v) bovine serum albumin (BSA) was used to wash cells and to dilute the antibodies.

To visualise the expression of 5T4 antigen, cells were fixed with 3.7% formaldehyde-PBS for 10 minutes and non-specific binding was blocked using normal rabbit serum for 10 minutes. The cells were then incubated with mAb 5T4 (1 μg/ml) or a mouse polyclonal anti-5T4 antiserum (Myers et al., 1994) for 1 hour, followed by fluorescein-conjugated rabbit anti-mouse immunoglobulins (1:40 dilution, Dakopatts) for 1 hour. The distribution of the 5T4 antigen was also examined following disruption of the cytoskeleton. Prior to immunofluorescence labelling, some cultures received cytochalasin D (10 μg/ml, Sigma), to disrupt the actin filaments, or demecolcine (10 μg/ml, Sigma), to disrupt the microtubules, for 2 hours. To ascertain whether live cells exhibited a similar pattern of staining with mAb 5T4 compared to fixed cells, some cells were labelled live at 4°C, using antibody solutions containing 0.1% azide, then post-fixed with 3.7% formaldehyde-PBS for 10 minutes. Appropriate controls with fluorochrome-labelled antibodies alone were included in each experiment.

The stained cells were examined and photographed using a Zeiss Axiophot inverted microscope equipped for epifluorescence. For confocal microscopy, cells were examined using a Zeiss laser scanning confocal microscope.

Electron microscopy

Cells to be analysed by transmission (TEM) and scanning (SEM) electron microscopy were cultured in 25 cm^2-base flasks, in MEMα containing 0.25% FCS. All procedures were carried out at 4°C. Cultures were washed with PBS. Some cultures were fixed with 1% paraformaldehyde-PBS for 20 minutes. In order to obtain maximal membrane preservation, some cultures were labelled unfixed. To visualise the expression of 5T4 antigen, the cells were incubated with normal goat serum (1:10 dilution in PBS/0.1% BSA) for 10 minutes followed by mAb 5T4 (1 μg/ml in PBS/0.1% BSA) for 1 hour. Cultures were then washed in PBS/0.1% BSA, followed by 50 mM Tris-buffered saline (pH 7.4) and then 20 mM Tris-buffered saline (pH 8.2), prior to incubation with 10 nm gold particle-labelled goat anti-mouse IgG (1:15 dilution in 20 mM Tris-buffered saline (pH 8.2), Amersham). Cells were subsequently prepared for TEM and SEM as described by Allen and Dexter (1982). Briefly, cells were fixed using 3% (v/v) glutaraldehyde in 0.1 M Sorensen's buffer (pH 7.4) for 1 hour, then post-fixed for 1 hour using 1% OsO_4 in 0.1 M Sorensen's buffer (pH 7.4). For SEM, selected areas of the flask were excised with a warmed cork borer. The cells on these discs of plastic were dehydrated, critical point dried, and coated with gold in situ before examination in a Topcon DS130 Field Emission Scanning Electron Microscope fitted with a KE Solid State Backscatter Detector. For TEM, the remaining cells in the flask were embedded in Luft's Epon. Sections were then stained with uranyl acetate and lead citrate prior to viewing with a Philips 400 transmission electron microscope.

Cell attachment

For assessment of cell attachment, 3×10^6 cells were seeded into wells of 35 mm diameter 6-well plates, in 2 ml of either serum-free MEMα containing 10 μg/ml transferrin, or MEMα containing 0.25% or 5% FCS. The numbers of attached and unattached cells (duplicate wells) were determined 24 hours later. Cell viability was determined by trypan blue exclusion.

Cell attachment to tissue culture plastic was compared with adhesion to components of the extracellular matrix. Fibronectin, vitronectin, laminin or collagen IV (10 μg/well in PBS, all Sigma) were added to wells of flat-bottomed 96-well microtiter plates and incubated at 37°C for 4 hours. Aliquots of 3×10^3 cells, in 100 μl of serum-free α-MEM containing 10 μg/ml transferrin, were then seeded into wells; 24 hours later, the cells were washed with PBS, and fixed in 70% (v/v) ethanol for 15 minutes. The fixative was removed and the plates air dried; 100 μl of 0.1% (w/v) crystal violet in 50 mM borate buffer (pH 9) was added to each well for 15 minutes. Excess dye was removed by extensive washing, the plates air dried. The A_{540nm} of each well was measured using an ELISA plate reader (Molecular Devices).

Cell aggregation assay

For assessment of cell-cell aggregation, 12.5 cm^2 vented cap flasks were coated with 2 ml aliquots of a 2.5% (w/v) solution of polyhydroxymethacyclate (Sigma) in 95% (v/v) ethanol and oven-dried at 60°C, to produce a non-adhesive surface. A total of 1×10^5 cells were seeded into coated flasks, in 2 ml of serum-free αMEM containing 10 μg/ml transferrin, or in MEMα containing 5% FCS. After 24 hours, the cells were examined with a phase-contrast microscope and cell aggregation determined by counting single cells and aggregates.

Motility and invasion assay

Cell motility was assessed using Falcon cell culture inserts with an 8 μm pore size PET membrane and cell invasive capacity using Biocoat MATRIGEL invasion chambers (24-well plates, Becton Dickenson Labware). The PET membranes used in these different assays were comparable. Conditioned medium was prepared by adding MEMα medium containing 0.25% FCS to a 75 cm^2 flask containing a 70% confluent culture of Balb/c 3T3 cells for 2 hours. Aliquots (0.5 ml) of fresh MEMα medium with 0.25% FCS, or conditioned medium, were added to the lower compartments of the chambers, and aliquots of 1×10^4 cells in 250 μl fresh medium were seeded into the upper compartments of the cell inserts. After 24 hours, the inserts were washed in PBS, fixed in 3.7% formaldehyde in PBS for 20 minutes and the cells stained by addition of 10 μg/ml Hoechst stain for 15 minutes. The membranes were removed with a scalpel blade and prepared for epifluorescence microscopy. Cell number on each side of the membranes was assessed by counting nuclei and, to account for differences in cellular attachment, results are expressed as the number of cells located on the lower side of the membrane as a percentage of the total number of cells.

RESULTS

5T4 cDNA-transfected L cells

The mAb 5T4 recognizes a 5T4 antigenic determinant that is conformationally dependent on both protein and carbohydrate moieties. Thus recognition of the cDNA product requires expression in a eukaryotic system allowing post-translational modifications. Two neomycin-resistant clones were produced by transfection of the murine A9 cell line with 5T4 cDNA subcloned into the expression vector pCMVαneo. Southern blot analysis of the A9-5T4a and A9-5T4b clones revealed that each had approximately 25 copies of the 5T4 gene per haploid genome. Western blot analyses (Fig. 1, Table 1) of these clones confirmed the expression of mAb recognized bona fide 72 kDa

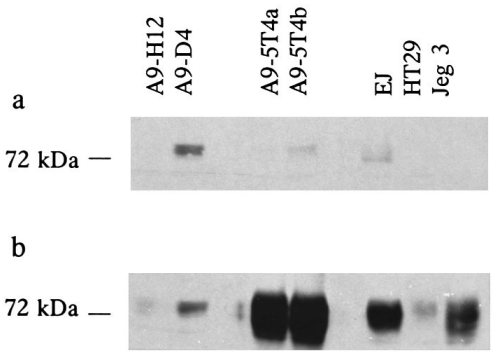

Fig. 1. Western blot analysis of A9-H12, A9-D4, A9-5T4a, A9-5T4b, EJ, HT29 and Jeg3 cell lines. Cells were lysed and separated by SDS-polyacrylamide gel electrophoresis using 10% (w/v) acrylamide and 0.1% SDS. Proteins were transferred to nitrocellulose using a Multiphor Novoblot electrophoresis transfer system. The 5T4 antigen was labelled with mAb 5T4 and detected by ECL. (a) Control, with no primary antibody; (b) mAb 5T4 antibody.

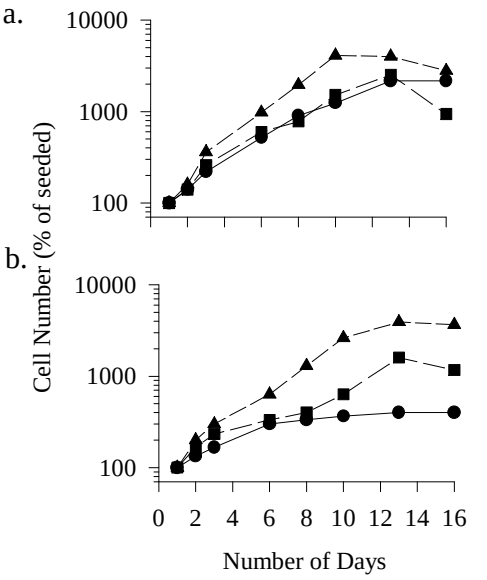

Fig. 2. 5T4 expression leads to decreased proliferation. (a) Clone A9-H12 control; and (b) clone A9-5T4a cells were seeded into wells of 6-well plates in serum-free medium. At 24 hours later, termed day 1, cells received either fresh serum-free medium (●) or medium containing 0.25% FCS (■) or 5% FCS (▲). At this time, day 1, and at further time-points up to 16 days, cell number was counted.

5T4 molecules. The two clones of neomycin-resistant vector alone-transfected cells were 5T4 negative. Analyses of HT29, EJ and Jeg3 cell lines confirmed the presence of native 5T4 molecules, although levels were lower compared with the 5T4-transfected A9 cells.

Altered cell morphology and division patterns in 5T4-transfected cells

Cells were routinely cultured in MEMα containing 0.25% FCS. This concentration of FCS was chosen, since it was the minimum that allowed growth of all cell clones. A low concentration was desirable, since factors present in FCS were observed to override some of the cell biological effects resulting from the presence of 5T4 molecules, including effects on cell proliferation (Fig. 2) and attachment (as discussed below). When cultured in serum-free medium, the doubling time of control A9-H12 cells was 62 hours (Fig. 2a). By contrast, little proliferation was observed with the A9-5T4a (Fig. 2b) or A9-5T4b (not shown) cells. This effect could be overcome by addition of FCS to the medium. Increasing the concentration of FCS to 0.25% resulted in only a slight increase in proliferation of the control clone cells, with a reduction in doubling time to 56 hours. A9-5T4a and A9-5T4b clone cells when cultured in this medium did proliferate, though with longer doubling times, 120 hours and 95 hours, respectively. Increasing the concentration of FCS to 5% increased the proliferation of all the cells, but the effect was most pronounced with the 5T4-transfected cells.

Phase-contrast (Fig. 3) and time-lapse video (Fig. 4) microscopy of both clones of 5T4-transfected A9 cells exhibited a more dendritic morphology (Fig. 3c,d; Fig. 4b) compared with the vector-transfected control cells (Fig. 3a,b; Fig. 4a). The 5T4-transfected cells also appeared more motile than the control cells (Fig. 4).

Division patterns of the clone A9-5T4a and A9-H12 control cells were determined (Table 2, Fig. 4). The time for division was measured from the start of rounding up until the cell had respread post-division. Clone A9-5T4a cells took approximately three times longer to pass through a division than control A9-H12 cells. Seventy six per cent of the time differ-

Table 1. Western blot analysis of 72 kDa 5T4 antigen: comparison of levels of expression in control and 5T4-transfected A9 cells with levels in the natural 5T4-expressing EJ, HT29 and JEG 3 cell lines

Cell line	Densitometry reading (arbitrary units)
A9-H12 (control)	982
A9-D4 (control)	124
A9-5T4a	20878
A9-5T4b	21187
EJ	10320
HT29	5869
Jeg 3	16542

Results are expressed as the difference in densitometry readings between the mAb 5T4- and control-immunolabelled bands.

Table 2. Time courses of division of cells from the control A9-H12 and 5T4-transfected clone A9-5T4a cells, determined by analysis of time-lapse video microscope images

Cell type	*n**	Division time† (minutes)	Spreading time‡ (minutes)	Total time§ (minutes)
Control A9-H12	7	57±8	69±11	127±15
Clone A9-5T4a	10	99±16	299±51	398±63

**n* represents the number of cells analysed.

†Time to divide, determined from the time of the start of rounding up until the time two cells separate. Data are expressed as mean±s.d.

‡Time to spread, determined from the time of division until the time the cells appear fully spread. Data are expressed as mean±s.d.

§Total time. Data are expressed as mean±s.d.

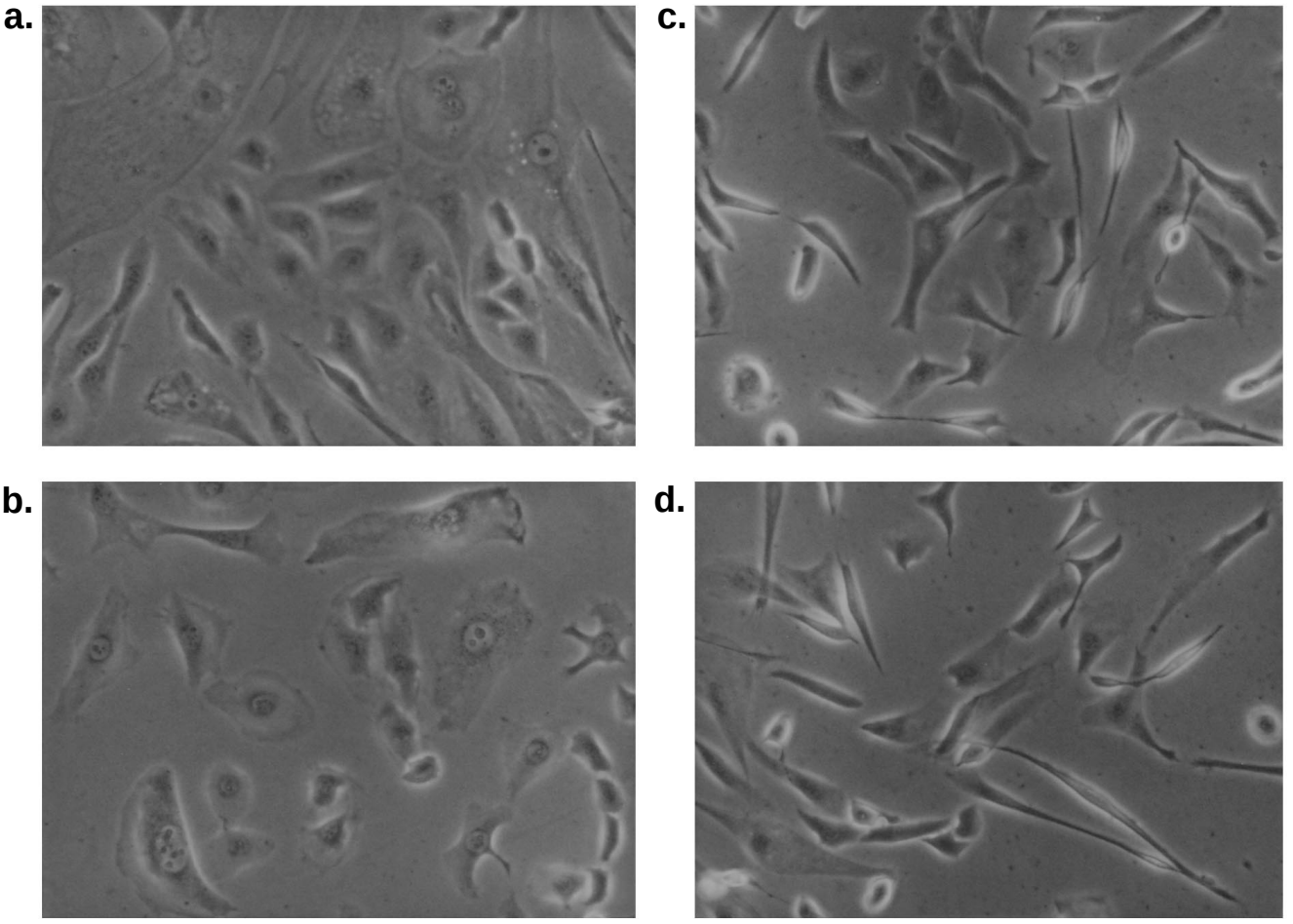

Fig. 3. Phase-contrast micrographs of vector alone- (a and b) and 5T4-transfected (c and d) A9 cells, cultured in MEMα containing 0.25% FCS. (a) A9-H12; (b) A9-D4; (c) A9-5T4a; and (d) A9-5T4b cells.

ence was accounted for by the time taken for the cells to reattach and spread following division.

5T4 antigen is not uniformly distributed in the plasma membrane

Using immunofluorescence microscopy, staining for 5T4 antigen was observed in populations of cells from each of the 5T4-transfected clones, although the intensity of staining between cells was heterogeneous (not shown). By confocal microscopy, individual cells exhibited a 'polkadot' pattern of staining, distributed over the entire plasma membrane. This pattern was found on both prefixed (Fig. 5a) and live cells (Fig. 5b), indicating that this pattern was not due to an antibody-induced redistribution. Similar results were obtained using monoclonal mAb 5T4 antibody (Fig. 5) or mouse polyclonal anti-5T4 antiserum (not shown). The 5T4 antigen was not concentrated at focal contact points.

Similar polkadot patterns of staining were observed in several human carcinoma cell lines which naturally express 5T4, including the EJ bladder carcinoma line (Fig. 5C), the HT29 colon carcinoma line, and the Jeg3 and JAR choriocarcinoma lines (not shown). In concurrence with the western blot analysis, the overall staining of the naturally 5T4-expressing cell lines was reduced in intensity compared to the 5T4-transfected A9 cells.

Since several cell surface molecules are known to associate with the cellular cytoskeleton, the effects of disruption of the cytoskeleton on the distribution of 5T4 antigen was examined (Fig. 6). Disruption of the microtubule network using demecolcine led to rounding up of the cells but the polkadot pattern of staining was conserved (Fig. 6b). Treatment of the cells with the actin filament-disrupting agent cytochalasin D resulted in the formation of large clusters of 5T4 staining (Fig. 6c).

5T4 is located at microvillus projections of the plasma membrane

The surface reliefs of cells from the control clone A9-H12 (Fig. 7a), the 5T4-transfected clones A9-5T4a (not shown) and A9-5T4b (Fig. 7b), and the natural 5T4-expressing EJ (Fig. 7c) cell line were studied by SEM. Control clone cells were spread, with numerous plasma membrane microvillus projections and some blebs. 5T4-transfected A9 cells appeared retracted and their plasma membrane surface reliefs exhibited numerous microvillus projections, blebs and surface folds. Similarly, the plasma membrane of the EJ cell line was characterised by numerous microvillus projections and blebs.

The relationship between the plasma membrane relief and 5T4 expression was then investigated, by TEM and SEM. TEM analysis of clone A9-5T4a cells revealed the presence of 5T4 antigen at the microvillus projections and blebs of the

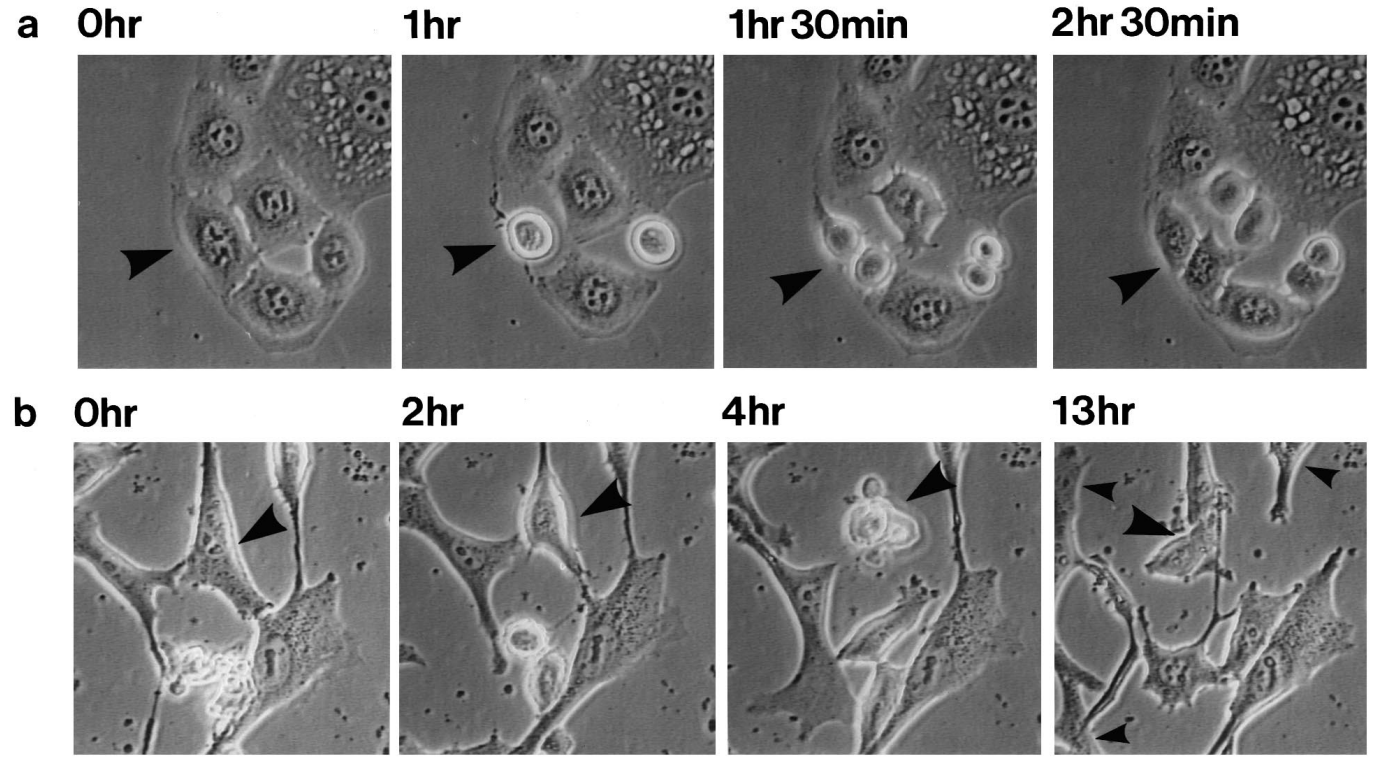

Fig. 4. Time-lapse video microscope images tracking the behaviour the vector alone-transfected A9-H12 control and 5T4-transfected A9-5T4a cells. (a) A9-H12 cells appeared pavement-like and little movement of cells was observed over 24 hours. The large arrowheads indicate cells which underwent their division cycle over 2 hours and 30 minutes. (b) A9-5T4a cells were more dendritic, and appeared more motile. The small arrowheads indicate examples of cells which had moved significantly over a 13 hour period. The large arrowheads indicate cells which underwent its division cycle over 13 hours.

plasma membrane, with little expression between (Fig. 8a,b). For SEM analysis, 5T4-transfected A9 cells were either fixed in 3.7% formaldehyde-PBS (Fig. 9a,b) or left unfixed (Fig. 9c,d) prior to immunolabelling, which results in better preservation of the plasma membrane. The SEM images confirmed the results with TEM; 5T4 antigen was concentrated at microvillus projections and blebs of the plasma membrane, with a similar overall staining pattern in fixed and unfixed cells. A comparable pattern was also observed with preparations of the EJ cell line.

5T4 expression decreases cell-substratum attachment

In cell counting experiments, in serum-free medium, A9-5T4a and A9-5T4b cells exhibited 30% and 20% decreased attachment, respectively, compared with both clones of control cells, of which greater than 95% attached (Fig. 10). Addition of 0.25% or 5% FCS to the medium led to increased attachment of the 5T4-transfected cells (Fig. 10). Cell viability was determined to be greater than 95% in all experiments.

The attachment of vector alone- and 5T4-transfected A9 clones to various components of the extracellular matrix were then compared (Table 3). Compared with their attachment to tissue culture plastic, increased attachment of all the clones was observed with fibronectin and laminin (P<0.01), and there was little difference with vitronectin and collagen IV. With all components, 5T4-transfected A9 cells attached less well (range of 68-79%) than the vector alone-transfected cells.

5T4 expression has no effect on cell aggregation

Cell attachment to the plastic of culture flasks is eliminated when the surfaces of the flasks are coated with poly-hydroxymethacyclate. Since the cells can no longer attach, cell-cell aggregation is promoted. However, there was no difference in cell aggregation between control clone cells (Fig. 11a) and 5T4-transfected cells (Fig. 11b), when cultured in medium that was either serum-free or contained 5% FCS.

5T4 expression increases cellular motility but not invasivity

Cell motility was assessed using a cell culture chamber assay,

Table 3. A comparison of the attachment of control A9-H12 and 5T4-transfected A9-5T4a cells to various components of the extracellular matrix

Component*	A9-H12† (A_{540} nm)	A9-5T4a† (A_{540} nm)	A9-5T4a attachment (% of A9-H12 attachment)
Plastic	0.20±0.05‡	0.14±0.05	70
Fibronectin	0.39±0.06	0.30±0.04	77
Laminin	0.25±0.04	0.17±0.06	68
Vitronectin	0.20±0.02	0.15±0.02	75
Collagen IV	0.19±0.04	0.15±0.05	79

*Tissue culture plastic was coated with extracellular matrix components at 10 μg/ml.

†Results are data from A9-H12 control and A9-5T4a-transfected cells. Similar data were observed with A9-D4 and A9-5T4b cells.

‡Results are expressed as mean±s.d. from 3 experiments.

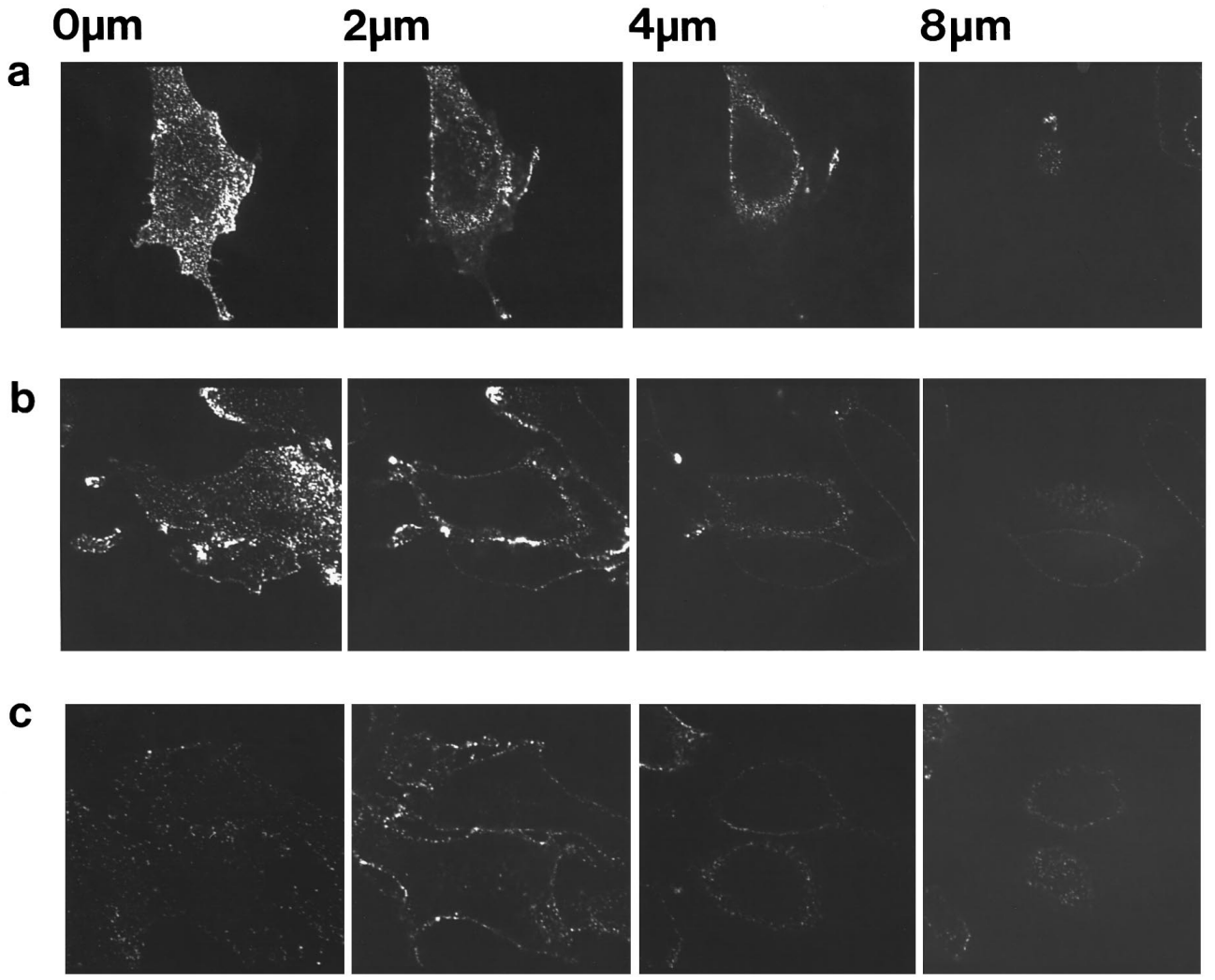

Fig. 5. Confocal microscope visualisation of 5T4 expression by clone A9-5T4a cells and the EJ cell line. 5T4 antigen on the plasma membrane of clone A9-5T4a cells and the EJ cell line, cultured on glass coverslips, was immunolabelled using mAb 5T4, fluorescein-conjugated rabbit anti-mouse immunoglobulins. Serial sections of the cells were visualised by confocal laser scanning microscopy. The section at 0 μm in each series is the point of contact with the glass coverslip. (a) Clone A9-5T4a cells, immunolabelled following pre-fixation. (b) Clone A9-5T4a cells, immunolabelled live and post-fixed prior to visualisation. (c) EJ cells, immunolabelled following fixation.

in which cells are seeded onto the upper side of a porous PET membrane. The ability of the cells to move to the lower side of the membrane is an indication of their motility. 5T4-transfected cells appeared twice as motile as control clone cells (Fig. 12). Since motility may be inducible by growth factors, Balb/c 3T3 conditioned medium was added to the lower compartment of the assay chambers but there was no differential increase in the motility of the 5T4-expressing cells compared to the control cells (Fig. 12).

Cellular invasion was determined using Biocoat MATRIGEL invasion chamber assay, in which cells must move through a basement membrane-like complex prior to crossing the porous PET membrane. A small, but not significant, increase in invasion was observed with the 5T4-transfected cells compared with control cells (Fig. 12). Addition of conditioned medium to the lower compartment of the assay chambers increased the invasivity of all the clone cells, but again no significant difference between the 5T4-transfected and control cells was observed (Fig. 12).

DISCUSSION

A9 cells transfected with 5T4 cDNA showed changes in morphology and behaviour which may reflect the function of 5T4 molecules in either the trophoblast of the placenta or tumour cells in their host. 5T4 antigen expression in A9 cells was associated with a more dendritic morphology, an altered pattern of division, decreased cell-substratum attachment and increased cellular motility.

Our previous observations that the extracellular domain of the 5T4 antigen contains seven LRRs had suggested a role for the glycoprotein in modulating cellular interactions. In the present study, no difference in homotypic cell aggregation was observed between 5T4- and vector alone-transfected A9 cells. However, several lines of evidence suggest that 5T4 antigen expression affects cell-substratum adhesion and cell motility. In the attachment assay, 5T4-transfected cells adhered to a number of substrata, including tissue culture plastic, fibronectin, laminin, vitronectin and collagen IV less than

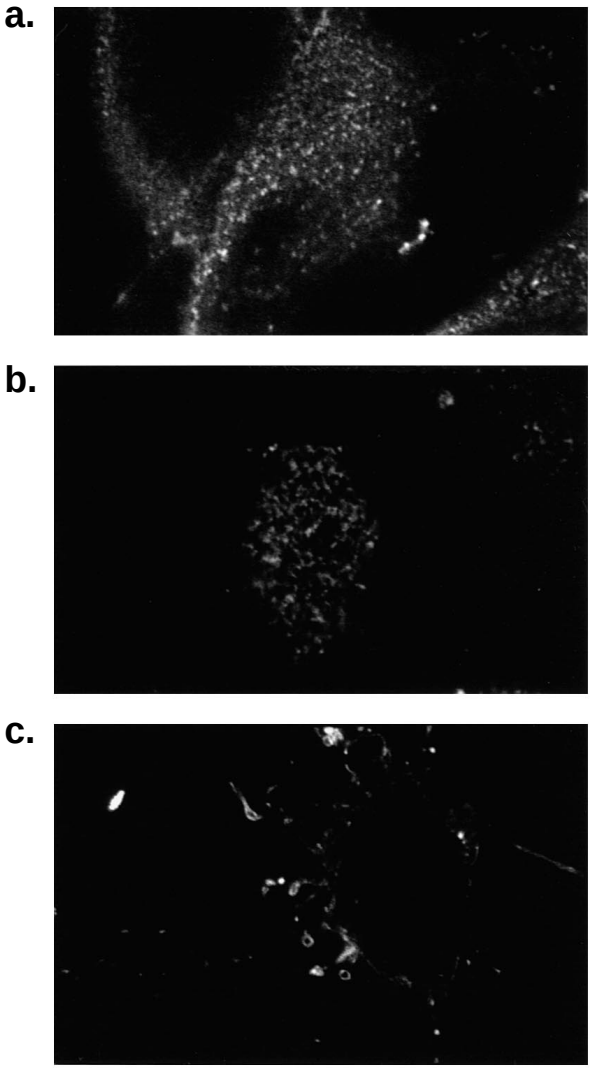

Fig. 6. Effects of cytoskeletal disruption on 5T4 expression by clone A9-5T4a cells. Clone A9-5T4a cells either received no pretreatment (a) or were pretreated for 2 hours prior to immunolabelling with demecolcine (b) or cytochalasin D (c) to disrupt microtubules or the actin cytoskeleton, respectively. Cells were then fixed in 3.7% formaldehyde-containing PBS and labelled using mAb 5T4 and fluorescein-conjugated rabbit anti-mouse immunoglobulins. Sections, at 0 μm, correspond to the point of contact with the glass coverslip.

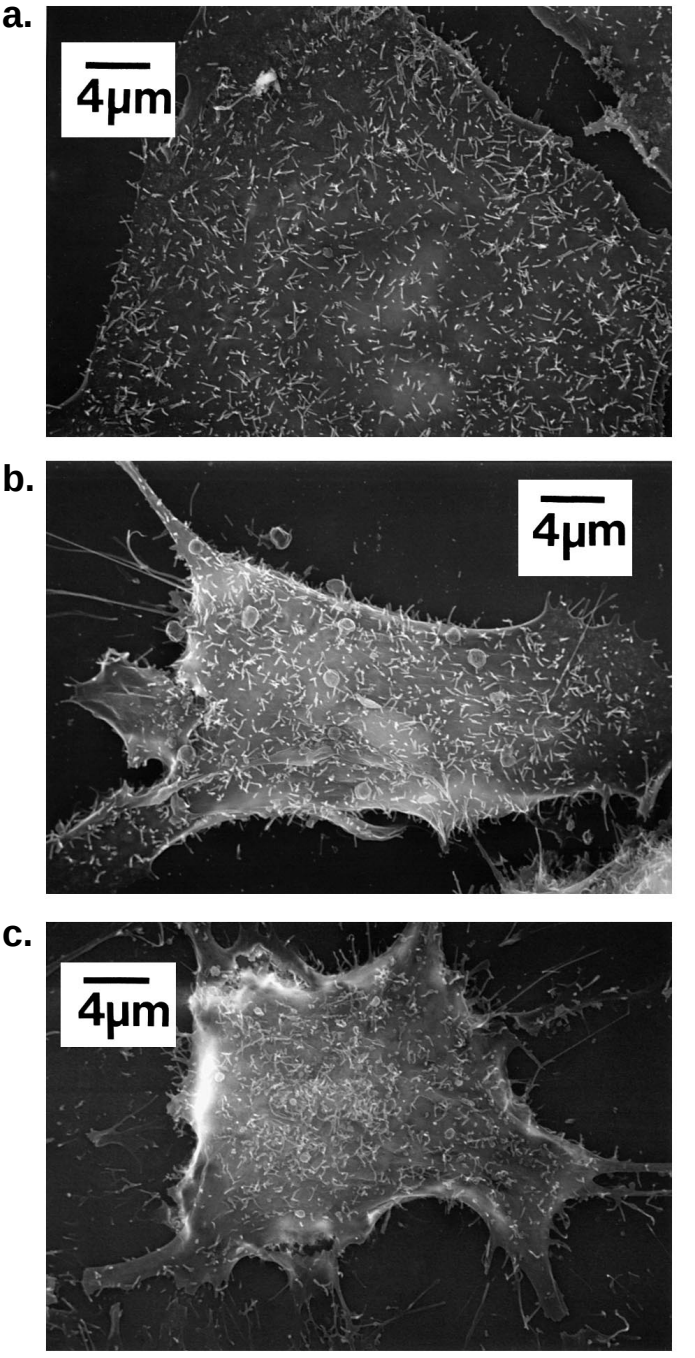

Fig. 7. Scanning electron microscope visualisation of the surface relief exhibited by the control clone, clone A9-5T4b and EJ cells. (a) Control clone cells appeared spread with numerous microvillus projections and few blebs of the plasma membrane. (b) Clone A9-5T4a cells appeared more retracted and exhibited numerous microvillus projections, blebs and surface folds. (c) The EJ cell line also exhibited numerous microvillus projections and blebs.

control cells. The most obvious alteration associated with 5T4 expression in A9 cells was the increase in cellular dendricity, a change consistent with reduced adhesiveness of the cells. An opposite effect has been reported following transfection of cultures of Schwann cells with the adhesion molecule syndecan-1 cDNA, where the cells changed in morphology from networks of small, bipolar cells with little spreading onto the substratum to highly flattened, cuboidal cells (Carey et al., 1994). The altered pattern of division and proliferation observed with 5T4 expression in A9 cells may also reflect decreased adhesiveness. The longer division times of 5T4-transfected A9 cells can be accounted for by the period of time taken to readhere and spread post-division. In these time-lapse studies, increased cellular motility was also observed following 5T4 expression in A9 cells; this was confirmed in the cell motility assays.

The finding that the 5T4 antigen is concentrated at microvillus projections and blebs of the plasma membrane further supports a functional role involving cell interactions. A microvillus projection may be considered as a subcellular

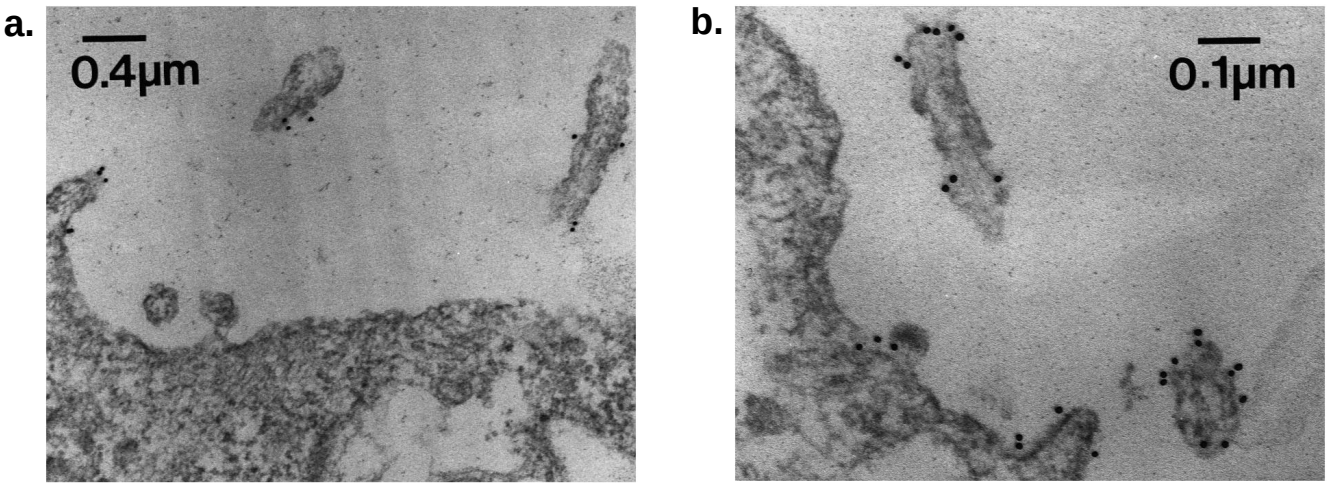

Fig. 8. Transmission electron microscope visualisation of 5T4 expression by clone A9-5T4b cells. Clone A9-5T4b cells were fixed using 1% paraformaldehyde, immunolabelled with mAb 5T4 and a 10 nm gold particle-labelled goat anti-mouse IgG, and prepared for TEM as described in Materials and Methods. Two typical sections are shown. 5T4 appeared localised at microvillus projections and blebs of the plasma membrane, with little expression in between.

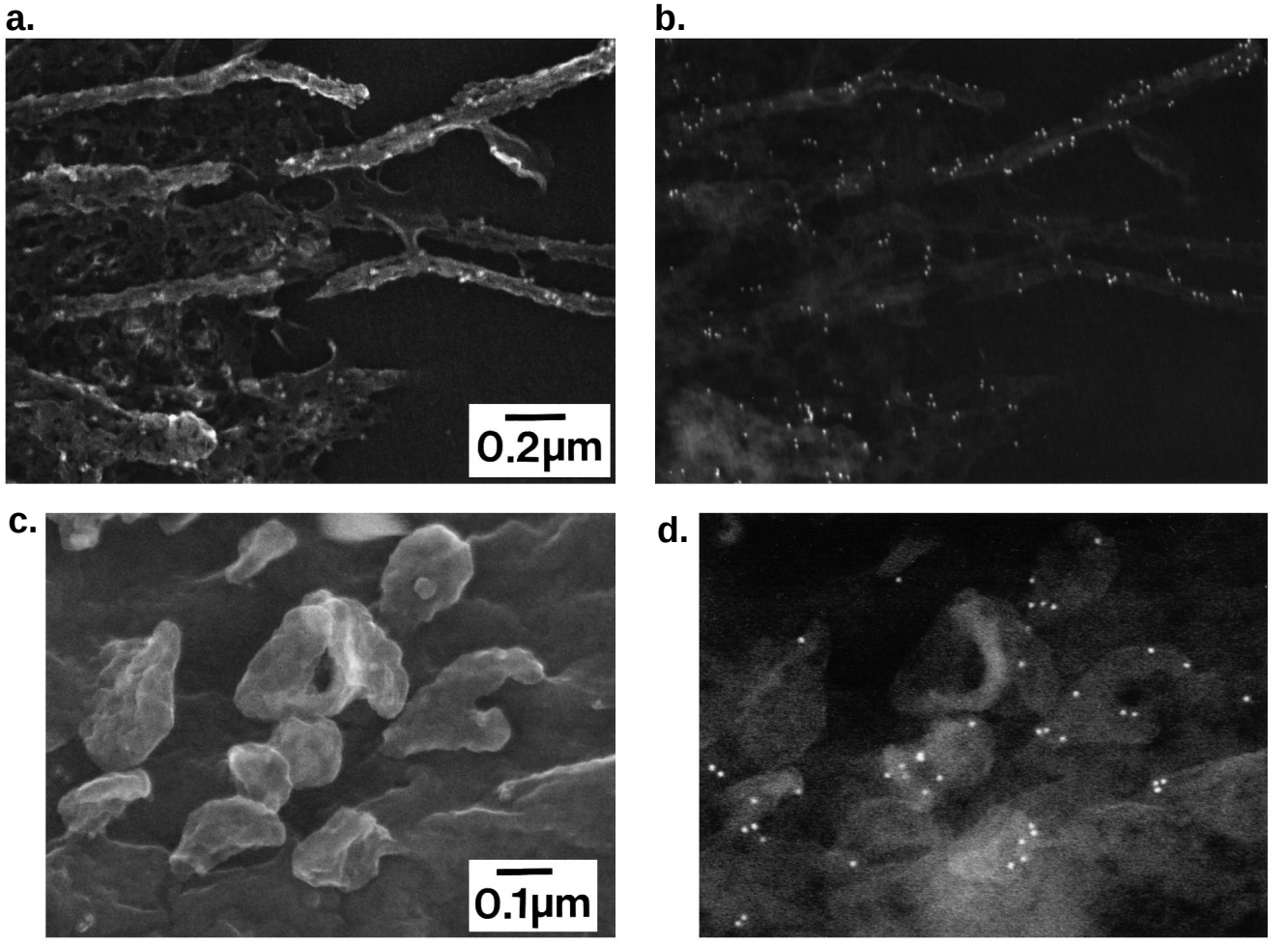

Fig. 9. Scanning electron microscope visualisation of 5T4 expression by clone A9-5T4a and clone A9-5T4b cells. Cells were immunolabelled with mAb 5T4 and a 10 nm gold particle-labelled goat anti-mouse IgG, and prepared for SEM as described in Materials and Methods. Cell surface relief (a) and gold particle immunolabelling of 5T4 antigen (b) of a clone A9-5T4a cell, fixed with 1% paraformaldehyde prior to immunolabelling. Cell surface relief (c) and gold particle immunolabelling (d) of a clone A9-5T4b cell, left unfixed during immunolabelling procedures in order to improve the preservation of the plasma membrane. In both cases, 5T4 appeared concentrated at microvillus projections and blebs of the plasma membrane.

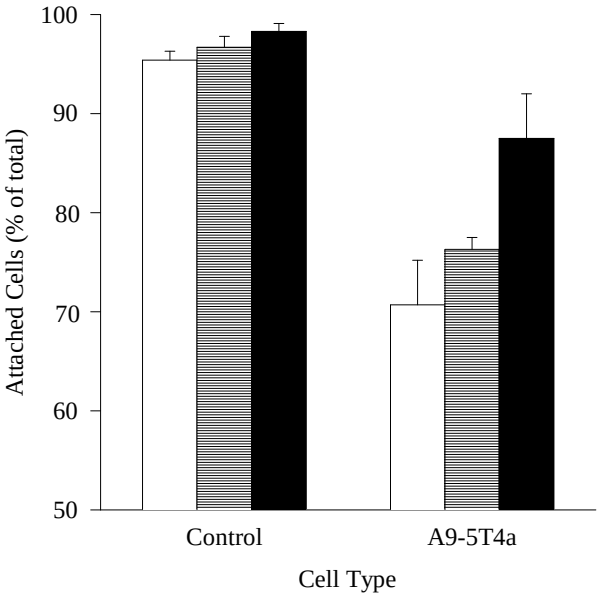

Fig. 10. 5T4 expression leads to decreased cell attachment. Control A9-H12 and 5T4-transfected A9-5T4a cells were seeded into wells of 6-well plates in serum-free medium (□), or medium containing either 0.25% (▤) or 5% (■) FCS. At 24 hours later, the number of attached cells, compared with the total number of cells, was determined. Data are the mean ± s.d. from 2 experiments.

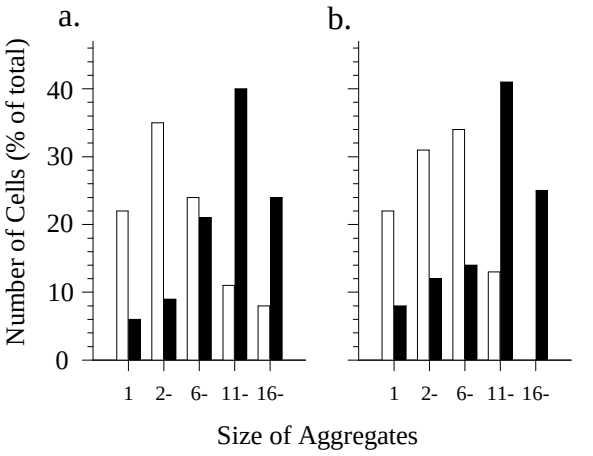

Fig. 11. 5T4 expression has no effect on cell-cell aggregation. Control A9-H12 (a) and 5T4-transfected A9-5T4a (b) cells were seeded into poly-hydroxymethacyclate-coated flasks in serum-free medium (□) or medium containing 5% FCS (■). At 24 hours later the numbers of cells in colonies were counted. Two fields of view, using a ×40 lens, were counted per flask and duplicate flasks were used for each cell line. Data are given as the percentage of cells in various colony size groups, and are the combined results from 2 experiments.

organelle, consisting of the plasma membrane, which contains a myriad of molecules involved in cellular interactions and a cytoskeletal core consisting of actin filaments. Rovensky et al. (1992) reported an increased number of microvillus projections in immortalised and transformed fibroblasts compared with normal cells. In this case, vector alone-transfected control A9 cells exhibit numerous microvillus projections. Thus the 5T4

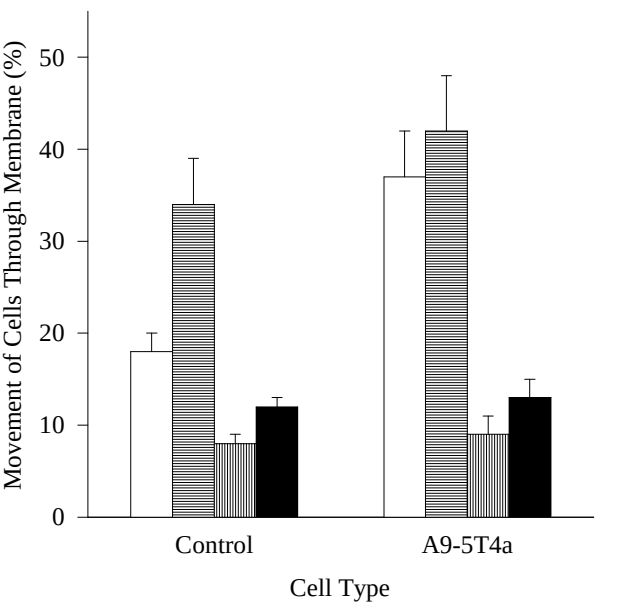

Fig. 12. 5T4 expression leads to increased cellular motility, but does not alone affect the invasive capacity of the cells. Control A9-H12 and 5T4-transfected A9-5T4a cells, in serum-free medium, were seeded into the upper chamber of cell culture inserts with an 8 μm pore size PET membrane (□,▤), or into MATRIGEL invasion chambers (▥,■). Fresh medium (□,▥), or 3T3 conditioned medium (▤,■), was added to the lower chambers. At 24 hours later the cells were fixed and then stained with fluorescent Hoescht dye. Cell numbers on each side of the membrane were assessed by counting Hoechst-stained nuclei, and the number of cells located on the lower side of the membrane was calculated as a percentage of the total number of cells. Results are the mean ± s.d. of at least 2 experiments for each condition. Duplicate membranes were prepared for each experiment, and for each membrane the cells in 4 fields, using a ×40 lens, were included. Similar patterns were observed in the control A9-D4 and the 5T4-transfected A9-5T4b cells.

antigen is not promoting microvillus projection formation per se; instead, expressed molecules are recruited to the projections. It may be argued that transfection of the cDNA of molecules into naturally non-expressing cells may give rise to an expression pattern and a resulting cell phenotype which does not reflect the true function of the molecules in their natural environment. However, 5T4 antigen is associated with microvillus projections in several carcinoma cell lines which naturally express the molecules including the EJ bladder-, HT29 colon- and the Jeg3 and JAR choriocarcinoma cells lines.

For some cellular functions, linkages between transmembrane molecules and cytoskeletal elements are required (Pavalko and Otey, 1994). For example, the adhesion molecule ICAM-1 localises at microvillus projections and associates with the actin-containing cytoskeleton via a direct interaction with the actin-binding molecule α-actinin (Carpen et al., 1992). CD44 is another actin-associated glycoprotein, but the identity of a cytoskeletal link protein remains elusive (Tarone et al., 1984; Lacy and Underhill, 1987; Neame and Isacke, 1993). The redistribution of the 5T4 antigen observed following treatment with cytochalsasin D suggests a link with the actin cytoskeleton. However, further work is required to determine whether

there is a true functional interaction between 5T4 molecules and components of the actin-related cytoskeletal network.

Several molecules have been reported as negative modulators of cell adhesion. Some are membrane-bound including: polysialated N-CAM, which antagonises cell-substratum attachment and cell aggregation of neuronal cells (Acheson et al., 1991); CD43, which has been observed to inhibit adhesive interactions between leukocyte function-associated antigen 1 molecules and ICAM-1 on CD43-transfected HeLa cells (Ardman et al., 1992); and CD34, whose expression is regulated reciprocally with other adhesion molecules in vascular endothelial cells (Delia et al., 1993). However, some negative modulators of cell adhesion are not expressed at the cell surface but secreted into the local environment where they are retained and exert their anti-adhesive function; examples include the glycoproteins SPARC, tenascin and thrombospondin (reviewed by Sage and Bornstein, 1991). Interestingly, in colorectal and gastric carcinoma tissue (Starzynska et al., 1992, 1994) two distinct phenotypes of immunohistochemical labelling with mAb 5T4 are observed; either the malignant cell membranes and surrounding stroma are positive, or reactivity is limited to the stroma adjacent to the tumour. The hypothesis currently being tested is that the stromal form of the 5T4 antigen is produced and secreted by the tumour cells. However, there are precedents for the induction of stromal glycoproteins produced by stromal cells, including the proteoglycan chondroitin sulphate, and decorin, an LRR-rich proteoglycan (Iozzo and Cohen, 1994).

The results of the present study support the hypothesis that 5T4 molecules may have a direct function in trophoblast and tumour cell invasion processes through interactions with the extracellular substratum. However, in A9 cells 5T4 antigen expression alone was not sufficient to induce invasion through MATRIGEL, a basement-membrane-like complex. It is possible that the 5T4 antigen may interact with other factors to increase invasion. For example, metalloproteinases, such as collagenase, may be needed to break down the extracellular matrix. The decreased adhesion and increased motility conferred by the 5T4 antigen may then result in a more invasive phenotype.

The authors are grateful for the technical assistance of Nigel K. Smith and Sandra A. Rutherford. This work was supported by the Cancer Research Campaign.

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(Received 7 March 1995 - Accepted 16 May 1995)