Sodium channel protein expression enhances the invasiveness of rat and human prostate cancer cells

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Abstract Expression of Na^+ channel protein was analysed in established cell lines of rat and human prostatic carcinoma origin by flow cytometry using a fluorescein-labelled polyclonal antibody. In many cell lines examined, the obtained frequency distribution profiles were bimodal and identified a subpopulation of cells which expressed high levels of Na^+ channel protein. A significant positive correlation was demonstrated between the proportion of channel-expressing cells and the functional ability of individual cell lines to invade a basement membrane matrix in vitro. In addition, two transfectant cell lines containing rat prostate cancer genomic DNA were found to express significantly elevated levels of Na^+ channel protein when compared with the original benign recipient cell line. Enhanced Na^+ channel expression by two metastatic derivatives of these transfectant cells directly correlated with increased invasiveness in vitro. These studies strongly support the hypothesis that expression of Na^+ channel protein and the metastatic behaviour of prostatic carcinoma cells are functionally related, either by endowing the membranes of these cells with specialised electrophysiological properties (e.g. enhancing their motility and/or secretory activities) and/or by perturbing endogenous mechanisms regulating ionic homeostasis within the cells.

Key words: Prostatic carcinoma; Flow cytometry; Na^+ channel; Metastasis; Tissue culture

1. Introduction

Voltage-activated Na^+ channels have been implicated in a range of cellular activities including proliferation, migration, adhesion and the cell cycle in addition to their recognised role in neural conduction and muscle contraction [1]. In prostatic carcinoma, recent evidence has suggested that the number of Na^+ channels expressed within the plasma membrane may be related to metastatic potential. Electrophysiological patch-clamp experiments revealed Na^+ channels to be present in approximately 47% of highly metastatic MAT-*LyLu* cells of rat but absent from the related but poorly metastatic AT2 cells [2]. In the former, the electrical activity of plasma membrane-located Na^+ channels, and the ability of the cells to invade basement membrane matrix in vitro, was inhibited by tetrodotoxin, a specific blocker of voltage-gated Na^+ channel activity [2]. These findings were supported by similar experiments on analogous human prostate carcinoma cell lines LNCaP and PC3. The former, which is not metastatic in nude mice, expressed no Na^+ channels, as detected by electrophysiology, Western blotting or flow cytometry. In contrast, approximately 10% of cells from the highly metastatic PC3 cell line expressed functionally active Na^+ channels which could also be detected by flow cytometry of cells labelled with a fluorescent antibody to Na^+ channel protein [1].

In the present study, we have employed flow cytometry to measure expression of Na^+ channel protein in a large series of behaviourally distinct prostatic epithelial cell lines. Seven of these (Table 1) were variants of the Dunning rat prostate carcinoma model which display a broad range of metastatic activity from $<5\%$ to $>75\%$ when inoculated subcutaneously into syngeneic rats [3]. We also examined three cell lines originally isolated from human prostate carcinoma metastases and one derived from human non-neoplastic prostatic epithelium. In each case, an important hallmark of malignancy, namely the ability to invade the basement membrane, was assessed by an in vitro invasion assay. In this way we aimed firstly to determine whether the level of Na^+ channel protein expression correlated with metastatic potential of prostatic carcinoma cells.

A second aim of the study was to determine whether conversion of cells from a benign to a malignant phenotype is accompanied by Na^+ channel protein expression. We have employed flow cytometry to analyse a series of transfectant cell lines containing rat prostate cancer genomic DNA sequences that we have recently developed [4]. These cells were specifically selected for their metastatic behaviour when inoculated into syngeneic hosts. A final aim of the investigation was to determine whether cells expressing Na^+ channels might represent a distinct population with an immutable phenotype, or whether they exist in equilibrium with their non-expressing neighbours. This involved using the sorting facility of the flow cytometer to separate cells of high Na^+ channel expression from the remainder and then analyzing the two fractions independently.

2. Materials and methods

2.1. Cell cultures

Sixteen cell lines were grown for flow cytometric analysis and invasion assay. Of these, seven comprised sublines of the Dunning R3327 rat prostate carcinoma originally isolated from a spontaneous tumour in a rat [3,5]. These cell lines (Table 1) have been well characterised and display metastatic activity in syngeneic Copenhagen male rats ranging from $<5\%$ to $>75\%$ [3,6,7].

2.2. Human cell lines

Four cell lines of human prostate were also used, three of which (Du145, LNCaP and PC3) were derived from human prostatic carcinoma metastases [8–10]. The fourth, PNT2, was derived from normal

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human prostatic epithelium immortalised by transfection with SV40 replication defective genome [11,12]. These, and the Dunning cell lines, were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% fetal calf serum (FCS), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Gibco) and 0.05 μg/ml each of hydrocortisone and testosterone (Sigma).

2.3. Transfectant cell lines

Four novel cell lines were previously generated in this laboratory by transfecting genomic DNA from rat prostatic tumour cells into a recipient benign cell line [4]. RMP1 cells were obtained by transfecting DNA from Dunning rat prostate G cells into rat benign mammary epithelial cell line RAMA 37 [13]. A subline of malignant cells derived from RMP1, designated RMP1a-*lu*, was generated by inoculating the parental cells into the mammary fat pad of Wistar-Furth rats and culturing cells isolated from a subsequent pulmonary metastasis [4]. RMP2 was produced by transfecting genomic DNA from Dunning AT6.1 into RAMA 37 cells. Thereafter, the RMP2c-*lu* subline was isolated from a pulmonary metastasis derived from the same cells inoculated into rats [4]. The recipient cell line, RAMA 37, was also grown as a non-malignant control with which the transfectant cells could be compared. All five of these cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 8 mM L-glutamine, 0.05 μg (1370 IU)/ml insulin, 0.05 μg/ml dexamethasone and the above antibiotics. All cell lines were kept at 37°C in a humidified atmosphere of 5% CO_2 and air. Medium was changed thrice weekly and cells harvested when approximately 75% confluent.

2.4. Na^+ channel antibody

The antibody used to label cells was a rabbit immunoaffinity purified polyclonal IgG fraction (Upstate Biotechnology Inc.) raised against a 19 residue synthetic peptide corresponding to a highly conserved intracellular loop of vertebrate Na^+ channel [14].

2.5. Flow cytometry

Viable cells were used for flow cytometric measurement of Na^+ channel protein expression. Tissue culture monolayers were released from 14 cm diameter culture dishes using 0.03% trypsin phosphate buffered saline (PBS) containing 0.02% EDTA for 4 min at 37°C after which the cells were transferred to a centrifuge tube and gently agitated by pipetting to obtain a single-cell suspension, which was confirmed microscopically. This ensured that a monodisperse suspension of cells was produced whilst reducing the contact with trypsin to a minimum. The activity of trypsin was then arrested with FCS, the cells washed in medium, resuspended in complete medium and an aliquot counted using a haemocytometer.

A volume of cell suspension containing 1×10^6 cells from each cell line was transferred to separate 1.5 ml microfuge tubes, the cells washed with 1 ml of PBS and supernatants replaced with 200 μl of blocking buffer comprising PBS containing 1% bovine serum albumin and left on ice for 10 min. To each supernatant was added 100 μl of Na^+ channel antiserum (Upstate Biotechnology Inc.) from a stock solution containing 12.5 μg protein per ml of blocking buffer. Negative control tubes containing the same number of cells received 100 μl of either blocking buffer alone or antibody preabsorbed with an excess of immunising peptide (Upstate Biotechnology Inc.). Both sets of tubes were incubated on ice for 1 h with mixing every 15 min. After centrifuging, the antiserum was withdrawn, the cells washed with 1 ml of PBS and 100 μl of anti-rabbit (goat IgG)-FITC conjugate, diluted 1:160 in blocking buffer was added to each of the tubes, including controls. Incubation occurred for a further hour on ice after which the supernatants were replaced with PBS. Fluorescence intensity was measured using a Becton Dickinson FACSort flow cytometer (San Jose, CA, USA) and analysed using Lysis software. Sensitivity of the instrument was adjusted so that the mean intensity of the negative controls fell within the first log decade with, at most, 5% lying above this range. Preabsorption of the antibody with antigen quenched all activity so that readings from these samples were closely similar to those in which the primary antibody had been omitted altogether. A total of 20 000 events were counted for each sample with electronic noise and sub-cellular debris excluded by setting a threshold on forward light scatter (representing cell volume). This procedure was performed on each cell line in two separate analyses and the average value calculated. Data were recorded as frequency distribution histograms, mean fluorescence intensity and the percentage of cells with an intensity greater than that of 95% of controls (proportion of cells positive for Na^+ channels). Suitable fluorescence histograms were subjected to a deconvolution programme. This was achieved by applying an equation of the algebraic sum of the two Gaussian distributions utilising data lying between the peak of the larger, left-hand population and the end of the tail of the secondary population. A curve-fitting routine [15] using the Levenberg-Marquardt algorithm [16] adjusted the parameters to minimise the value of chi-square between the computed and the actual histograms. The procedure was halted when the difference between approximations was less than 0.01.

2.6. Invasion assays

The ability of each of the cell lines to invade a basement membrane matrix was measured using an established in vitro invasion assay [1,2,17]. Extracellular matrix (ECM, Sigma) was diluted 1:2 with DMEM and 15 μl applied to the membrane of 12 mm diameter Millicell filter inserts with a pore diameter of 12 μm (Millipore). The fluid was spread evenly over the upper surface of the polycarbonate filter with a fine glass rod and the inserts heated to 37°C for 10 min to polymerise the gels which comprised 100 μg protein per cm^2 of filter surface.

Cells from each cell line to be analysed in the invasion assays were harvested with trypsin/EDTA and suspended in DMEM containing dialysed and stripped FCS (1% v/v) such that there were 6.2×10^4 cells per ml. 0.4 ml of this cell suspension (4.2×10^4 cells/cm^2) was dispensed into each Millicell filter insert in triplicate, the inserts placed in a 24-well multiwell plate (Nunclon) and each well filled with 0.6 ml of identical medium. The plates were incubated at 37°C for 48 h after which non-invading cells were wiped from the upper surface of the filters with a cotton bud. The dislodged cells were aspirated and replaced with 0.4 ml medium. The wells were filled with 480 μl of medium plus 120 μl of a solution containing 5 mg/ml of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma). The cells were incubated with MTT for 5 h after which the fluid was removed and the formazan dye dissolved with 400 μl of dimethyl sulphoxide at 37°C for 10 min. Aliquots of 195 μl were transferred in duplicate from each well to a 96-well microtitre plate and the optical absorbance measured with a Multiskan MS plate reader using a filter of 570 nm. A standard curve of optical absorbance against cell number for each cell line was constructed by seeding serially diluted cells of known number into a 24-well plate with DMEM and 1% stripped FCS and leaving them to attach for 24 h at 37°C. The MTT assay was then performed as described above. Optical absorbance of each triplicate invasion assay was converted into cell number by regression analysis of the linear part of the curve and the mean and standard deviation calculated.

2.7. Cell sorting

Two samples of 2×10^6 cells from each of AT3 and MAT-*LyLu* were labelled with antibody to detect Na^+ channel protein expression, as described (see Section 2.5) above. The cells were passed through the flow cytometer which had previously been sterilised with isopropyl alcohol followed by lengthy purging with sterile distilled water. The resultant frequency distribution histograms of fluorescence were bimodal and so a threshold was set to coincide with the trough between the peaks. Cells with a fluorescence intensity above this line were sorted from one sample of each pair, and cells with a lower intensity were collected from the other using the piezo electric sorting facility. The subpopulations of cells were grown to near confluence and subcultured at a split ratio of 1:4. When 75% confluent the cells were harvested, labelled with antibody to Na^+ channel protein and re-analysed flow cytometrically using an identical procedure.

3. Results

3.1. Dunning rat cell lines

Flow cytometric frequency distribution histograms of cells labelled for Na^+ channel protein expression in the seven phenotypically distinct cell lines of the Dunning rat prostate carcinoma model, together with their respective negative controls, are shown in Fig. 1. The two weakly metastatic cell lines (G and AT1) exhibited fluorescence profiles coincident

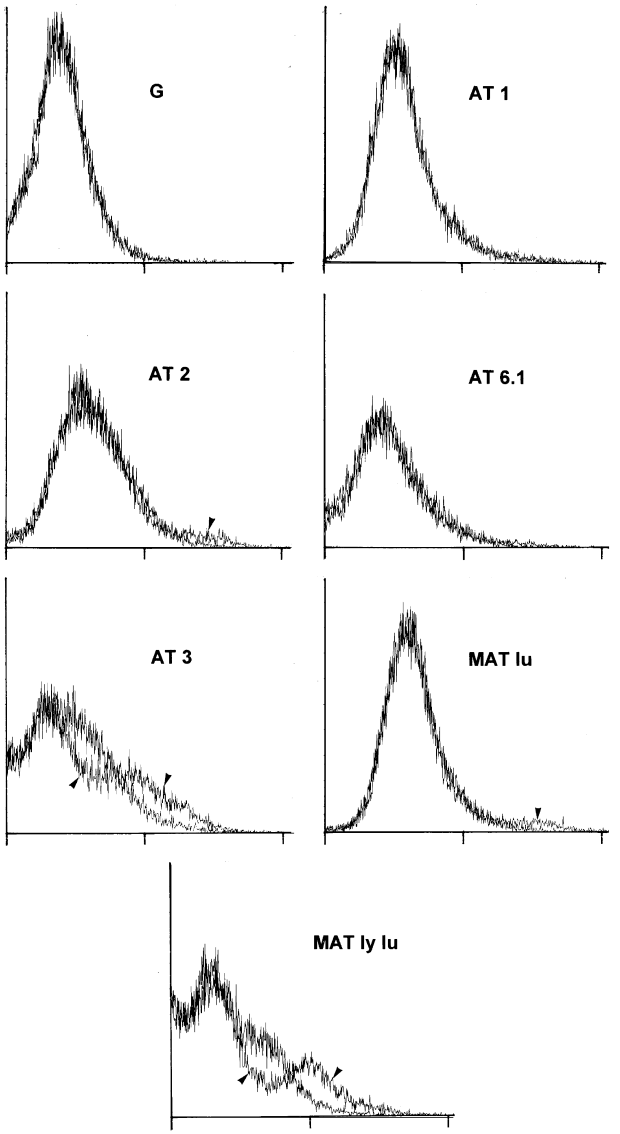

Fig. 1. Flow cytometric frequency distribution histograms of immunofluorescence for Na^+ channel protein from seven cell lines of the Dunning rat prostate carcinoma model. In each case, plots of both control and labelled cells are overlayed with the latter indicated by arrows where there is a clear distinction between the two lines. In these and Figs. 3 and 4, fluorescence intensity units are represented on the logarithmic abscissae which include two log decades and the numbers of cells are on the ordinate which spans 200 events.

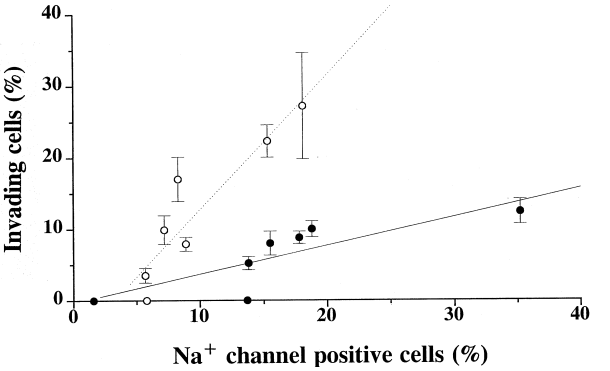

Fig. 2. Graphic plots showing the relationships between the percentage of invading cells versus the percentage of Na^+ channel-positive cells for the two different groups of cell studied ($n=7$, each). Open symbols/dotted line, the Dunning rat prostatic cancer cell lines; closed symbols/solid line, human prostate cancer cell lines and the RAMA 37 cell line transfected with rat prostate cancer cDNA (up to 12.4% invading cells). Linear regression analyses gave correlation coefficients of 0.97 for the Dunning cells; and 0.83 for the human prostate cancer and transfectant cell lines. The regressions were statistically significant for both groups of cells ($P<0.01$ and $P<0.05$, respectively).

with those of the controls. Cell line AT2, which has a low to moderate metastatic ability, exhibited a distribution pattern largely coincident with its control except for a small enhanced forward scatter of increased immunofluorescence. In contrast, analysis of the two highly metastatic cell lines (AT3 and MAT-*LyLu*) revealed distinct bimodal distributions (Fig. 1). The mode of the principal peak coincided with that of the controls while a secondary, smaller peak, represented a subpopulation of cells with a high level of Na^+ channel protein expression. The remaining two cell lines (AT6.1 and MAT-*Lu*) exhibited little difference from their respective controls (Fig. 1).

Since the modes of the larger frequency distribution peaks for both labelled and control cells were coincident, mean values of fluorescence intensity differed little between the seven cell lines. Therefore, these data have not been included. However, greater differences were revealed when the data were expressed as the *proportion* of cells with positive immunofluorescence (Table 1). Furthermore, a highly significant ($P<0.01$) positive correlation was found between the proportion of Na^+ channel immunopositive cells and the percentage of invading cells deduced from the in vitro invasion assay (Fig. 2).

3.2. Human cell lines

Data obtained from the four human prostate cell lines are

Table 1
Proportion of cells positive for Na^+ channels by flow cytometry and results of invasion assay for cells of the Dunning rat series

Cell line	Metastatic ability in vivo[a]	Percentage of positive cells	Percentage of invading cells	Secondary population as % of total cells[b]
G	None to low	5.8	0.0	–
AT1	Low	5.7	3.5 (1.02)	–
AT2	Low to moderate	8.3	17.0 (3.11)	5.1
AT6.1	High	7.2	9.9 (2.01)	–
AT3	High	15.3	22.3 (2.25)	35.2
MAT-*Lu*	High	8.9	7.9 (0.99)	–
MAT-*LyLu*	High	18.1	27.2 (7.40)	25.6

Figures in parentheses represent ± one standard deviation from the mean.
[a]From Isaacs et al. [3].
[b]As derived from deconvolution of frequency distribution histograms.

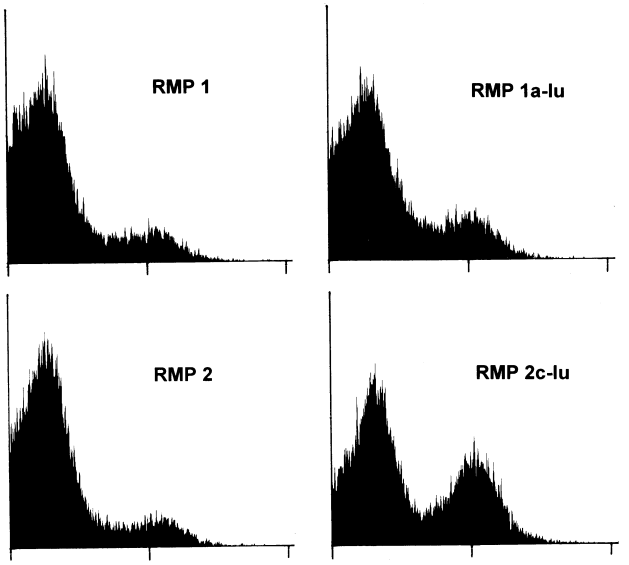

Fig. 3. Flow cytometric frequency distribution profiles from transfectant cell lines as described in the text. Cells derived by direct in vitro passage of the products of transfection, RMP1 and RMP2, both show bimodal distributions with a small secondary peak of positively fluorescent cells. Cells derived from pulmonary metastases of the transfectants in rats, RMP1a-*lu* and RMP2c-*lu*, are also bimodal but the secondary peak is enlarged suggesting an increased sub-population of cells expressing Na^+ channels. In each case the larger, left-hand peak was coincident with that of the controls (not shown).

shown in Table 2A. Of the three malignant cell lines, moderate Na^+ channel expression by both Du145 and PC3 cells was found whereas in LNCaP cells channels were scarce. Frequency distributions of immunofluorescence for Du145 and PC3 were both bimodal with a considerably larger secondary peak in the latter. These differences were reflected in their scores by invasion assay. The immortalised normal prostatic epithelial line PNT2 showed no ability to invade basement membrane matrix and yet 13.7% of its cells were immunopositive.

3.3. Transfectant cell lines

Flow cytometric frequency distributions for the cells transfected with DNA from Dunning rat prostate carcinoma cells are shown in Fig. 3. Profiles for the two cell lines derived from in vitro passage of the transfected cells (RMP1 and RMP2) were similar and showed prominent bimodal distributions.

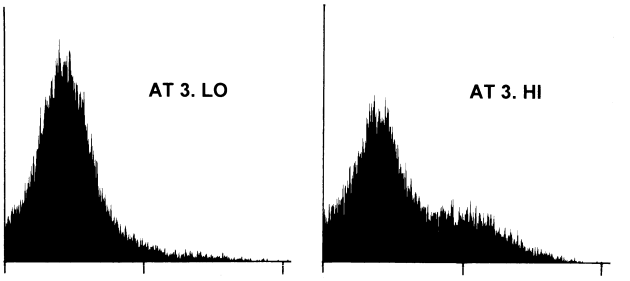

Fig. 4. Flow cytometric frequency distribution profiles of AT3 cells sorted into high and low fluorescence fractions, grown for several population doublings and then reassessed by flow cytometry. Descendents of the low fluorescence fraction (AT3 LO) produced a unimodal distribution with a narrow 'tail' of positive cells. The distribution for cells descended from the high fluorescence fraction (AT3 HI) is distinctly bimodal representing a large subpopulation of positive cells.

The two cell lines isolated from pulmonary metastases of the transfectants were also bimodal but the secondary peaks were larger suggesting that the size of the subpopulation of cells expressing Na^+ channels had increased.

Numerical data from these cell lines (Table 2) confirm that, in both instances, the proportion of immunopositive cells was enhanced in the metastatic lines when compared to their progenitors. Similarly, their capacity to invade basement membrane matrix was also increased. Taking the human prostate cancer and the transfected cell lines together, the data suggested that the correlation between the proportion of Na^+ channel immunopositive cells and the percentage of invading cells to be significant ($P = 0.05$) up to and including 12.4% of cells being invasive (Fig. 2). Accordingly, significant invarices would occur when more than 11.8% of the cells are Na^+ channel-positive.

3.4. Deconvolution of histograms

The frequency distribution histograms which showed a bimodal distribution were subjected to a deconvolution programme resolving them into two distinct histograms which could be analysed independently, as detailed in Section 2. The number of positive cells included in the secondary population is expressed as a percentage of the total cell population in Tables 1 and 2.

3.5. Cells sorted by flow cytometry

Flow cytometry was used to separate cells expressing high

Table 2
Proportion of cells positive for Na^+ channels by flow cytometry and results of invasion assay for human prostate cancer cell lines and transfectant cell lines

	Cell line	Percentage of positive cells	Percentage of invading cells	Secondary population as % of total cells[a]
A	PNT2	13.7	0.0	–
	LNCaP	1.6	0.0	–
	Du145	13.8	5.2 (0.93)	19.4
	PC3	18.8	10.0 (1.12)	45.6
B	RAMA 37	9.7	14.4 (1.61)	–
	RMP1	17.8	8.8 (0.88)	16.0
	RMP1a-*lu*	19.3	20.0 (4.48)	19.8
	RMP2	15.5	8.0 (1.70)	14.9
	RMP2c-*lu*	35.2	12.4 (1.74)	35.8

Figures in parentheses represent ± one standard deviation from the mean.
[a]As derived from deconvolution of frequency distribution histograms.

levels of immunofluorescence from those with low immunofluorescence for Na^+ channels in cell lines AT3 and MAT-*LyLu* of the Dunning rat series. For MAT-*LyLu* cells, the resulting frequency-distributions were similar and unimodal with long 'tails' of high fluorescence extending to the right. The proportion of positive cells was 8.8% from the low fluorescence fraction and 4.7% from the high fluorescence fraction. Frequency distributions from sorted AT3 cells were markedly dissimilar (Fig. 4). The distribution derived from cells with a low fluorescence was unimodal, whereas that from the high fluorescence fraction was bimodal with a large secondary peak (Fig. 4). The proportions of Na^+ channel-positive cells were 6.6% and 22.2%, (10.6% and 40.0% by deconvolution), respectively.

4. Discussion

This investigation has demonstrated that Na^+ channel proteins are expressed by malignant prostate cells which have a high metastatic ability. Our work not only extends the earlier findings of Grimes et al. [2] and Laniado et al. [1] to two additional Dunning rat cell lines and two human prostate carcinoma cell lines, but also provides new information relating expression of the Na^+ channel to specific genomic sequences derived from metastatic prostatic carcinoma cells.

By performing flow cytometry on a large series of cell lines, we have demonstrated that the proportion of cells expressing Na^+ channels correlates positively with their ability to invade basement membrane matrix in vitro. With respect to the seven cell lines of the Dunning rat series, the correlation was statistically significant only up to and including 27.2% of the cells being invasive. With respect to the human prostate carcinoma lines, the correlation was significant only up to 12.4% of the cells being invasive. Two of the human cell lines, Du145 and PC3, expressed positive Na^+ channel immunoreactivity and were highly invasive in vitro. Conversely, LNCaP is an androgen-sensitive cell line which does not metastasise in nude mice [8,18]. We found very few Na^+ channels in cells from this line and appropriately it scored zero on invasion assay, findings which concur with those of Laniado et al. [1]. However, the correlation between Na^+ channel expression and invasiveness was significant in a restricted range (0–12.4%) of cells becoming invasive. This would imply that higher levels of invasiveness may involve additional factors. Grimes et al. [2] and Laniado et al. [1] also found that blocking Na^+ channels with tetrodotoxin only partially suppressed invasion in vitro. Thus, our in vitro studies suggest that the presence of Na^+ channels in prostate carcinoma may act as a marker of metastatic ability.

The reason(s) for the observed discrepancies is not clear. In the case of PNT2, a transformed cell line derived from normal human prostatic epithelium which is reportedly benign but yet exhibited numerous Na^+ channels, transfection with the SV40 genome may confer upon the cells the ability to express Na^+ channels. Alternatively, PNT2 cells (and possibly other cell lines) may require more than the observed 13.7% of Na^+ channel expression to become invasive. Another cell line, RAMA 37, despite its benign nature, contained more cells with Na^+ channels than the malignant MAT-*Lu* line and also scored highly in the invasion assay. It may be that, although this cell line exhibits all the histopathological features of a benign tumour in vivo, a proportion of its constituent cells have the *potential* for invasion but are normally prevented from doing so by local factors. It is also the only line in our series which is entirely non-prostatic in origin. Another possibility is that while expression of active Na^+ channels may be a prerequisite of the metastatic phenotype, invasive and metastatic behaviour only occur in the circumstance that other essential criteria also have been fulfilled. In contrast to *phenotypic* appearances, which can be identified in a progressive sequence from normal to malignant and ultimately metastatic, genotypic abnormalities do not, necessarily, occur in a concomitant sequence [19]. Thus, expression of genes which ultimately trigger metastasis in prostate cancer, and possibly in other epithelial malignancies, may occur early in the phenotypic progression of a neoplasm but only become manifest with the accumulation of other mutations [20].

Flow cytometric frequency-distributions of fluorescence intensity were bimodal for those cell lines showing positive immunoreactivity, suggesting that cells which express Na^+ channels form a distinct subpopulation. This observation is at variance with that of Laniado et al. [1] who found no evidence for a subpopulation of PC3 cells since the entire flow cytometric profile was simply displaced to the right relative to the control. Data derived from deconvolution of the histograms indicate that the subpopulation expressing Na^+ channels accounts for as little as 5.1% of the total in AT2 cells to as much as 45.6% in PC3 cells. On the whole, these percentages are higher than those found by electrophysiology (0 and 10%, respectively), implying that a 'basal' level of Na^+ channels, below the threshold for detection by patch-clamp recording, exists in these cells.

We attempted to determine whether cells expressing high levels of Na^+ channel protein represent a distinct subpopulation with an immutable phenotype or whether these cells coexist in equilibrium with their Na^+ channel-negative neighbours by sorting the cells into high and low fluorescence fractions. If the cells are clonal in nature, flow cytometric histograms of the two fractions should reflect the phenotype of the originally sorted cells. This was confirmed for AT3 cells where the progeny of a high fluorescence fraction contained an increased proportion of positive cells whereas descendants of the low fluorescence fraction did not. However, it is likely that cells expressing Na^+ channels are not a true subclone of immutable phenotype otherwise all of the descendants of the high fluorescence fraction in AT3 would have been immunopositive (instead of only 22%). Nevertheless, it is of interest, and potential biological importance, that the cells apparently retain their phenotype for many divisions and only gradually revert towards their original proportions within the population.

Studies of the two transfectant cell lines, RMP1 and RMP2 indicate that when an epithelial cell is converted from benign to malignant by a single genetic event, the transformation is accompanied by spontaneous expression or upregulation of Na^+ channel protein. Of particular interest is the RMP1 cell line where the recipient cell (RAMA 37) is totally benign in vivo but contains moderate numbers of cells expressing Na^+ channels and the donor G cells is almost completely benign (metastasis frequency $< 0.1\%$) and expresses few Na^+ channels. The resulting transfectant line, however, is metastatic in vivo [4], invasive in vitro and contains a high proportion of cells expressing Na^+ channels. This finding suggests that the genes involved in invasion and metastasis of prostate cancer

cells are closely associated with those regulating expression of Na^+ channel proteins. However, the two transfectant cell lines (RMP1 and RMP2) were not cloned prior to these experiments and therefore, were likely to be of mixed genotype containing cells of differing metastatic potential. Inoculation of these genetically heterogeneous cells into syngeneic rats produced pulmonary metastases from which sublines RMP1a-*lu* and RMP2c-*lu* were derived. It was anticipated that this process would be selective for the metastatic malignant genotypes and this appears to be valid since both cell lines scored more highly on invasion assay and included more cells with Na^+ channels than their respective progenitors, particularly RMP2c-*lu*. This finding adds further credence to there being a close link between Na^+ channels and metastatic potential of prostate cancer cells. This postulated association complements the growing evidence that a range of ion channels play important roles in promoting the metastatic process, not only in prostate cancer but also in a wider range of epithelial malignancies. Thus, Ca^{2+}-binding protein p9Ka has been shown to be overexpressed in mammary cancers of both mice [21] and humans [22] and transfection of the p9Ka gene into previously benign rat mammary adenoma cells (RAMA 37) induces a metastatic malignant phenotype in a single step [23]. Recently we have shown that expression of p9Ka mRNA by cell lines of the Dunning rat prostate series increases proportionately with increasing malignant potential of the cell lines in a manner comparable with the Na^+ channel immunoreactivity described in this paper [24]. Similarly, expression of a newly recognised K^+ channel by cells of androgen-sensitive prostate cancer cell line LNCaP has been correlated with increased cell proliferation [25]. Thus, we believe that there is a growing body of evidence indicating that Na^+ channel protein expression may be a useful marker for metastatic potential in prostate cancer which, together with other ion channel proteins, may have a close functional association with the malignant phenotype as a promotional event in the development and progression of epithelial malignancies.

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