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NBT-II carcinoma behaviour is not dependent on cell–cell communication through gap junctions

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Abstract

To study the mechanism(s) underlying the proliferation of heterogeneous cell populations within a solid tumour, the NBT-II rat bladder carcinoma system was used. It has been first investigated whether the different cell populations are coupled through gap junctions (GJIC). Cells overexpressing the Cx43 were generated to test for any tumour suppressive activity in vivo. To determine whether GJIC is essential for tumour proliferation and the establishment of a cooperative community effect, NBT-II cells that are incompetent for cell coupling were generated. The data report that (i) carcinoma cells expressing or not FGF-1 are coupled through GJIC in vitro and in coculture and express the gap junction protein Cx43, (ii) overexpression of Cx43 in these cells does not affect their in vitro coupling capacities and in vivo tumourigenic growth properties, (iii) inhibition of GJIC through antisense strategy has no in vivo obvious consequence on the tumour growth properties of the carcinoma, and (iv) the community effect between two carcinoma cell populations does not critically involve cell coupling through gap junctions.

Keywords: Carcinoma; Connexin 43; GJIC; Antisense strategy; Cell–cell communication; Tumour proliferation; Community effect

Gap junctions mediate cell coupling via cell–cell communication in almost all tissues and participate in the complex process of homeostatic regulation [1,2]. Intercellular coupling allows coordinated cellular activity within a tissue through selective transfer of small metabolic molecules and second messengers. Connexins are transmembrane protein components of the gap junction channels and are concentrated at the cell-contact areas [3].

Transfer of growth regulatory signals through gap junctions is thought to be important in the control of proliferation and downregulation of connexin expression; poor gap junctional intercellular communication (GJIC) may be associated with uncontrolled cell growth and thus with neoplasia [4–7].

Several reports describe conflicting findings about the possible role of gap junctions in cancer progression. Nevertheless, many lines of evidence arising from recent work are consistent with connexins behaving like tumour suppressor genes [8–12]. In human and mouse lung carcinomas [13] and during tumour progression in various tissues [14–18] the neoplastic phenotype is frequently associated with low connexin expression. Transformed or tumourigenic cell lines transfected with connexin cDNAs exhibit restored GJIC and have proportionally reduced growth and tumourigenic potential in experimental models [19–21,10,11]. Conversely, in rat bladder carcinoma cell lines, high expression of connexin 43 and connexin 26 is correlated with the cells communicating, but having a strong tumourigenic potential [22]. Recently, it was reported that connexin 43 transfected-HeLa cells acquired invasive properties in vitro [23].

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Gap junctions or intercellular channels allow direct exchange of cytoplasmic substances between adjacent cells. Such coupling between adjacent cells of the same origin has been extensively studied. In contrast heterocellular gap junction communication remains poorly understood. GJIC is determinant for the inhibition of transformed cell growth by non-transformed cells [24] and is important in the transendothelial migration of invading cells [25] and of metastatic tumour cells [26]. In a recent report, heterocellular communication between Cx43 epithelial and fibroblastic expressing cells was shown to be almost absent, even after E-cadherin expression by the fibroblastic cells; however cell coupling can be obtained in vitro in triple cell coculture with cells of a transitional phenotype to bridge between epithelial cells and fibroblasts [27].

We have studied the mechanism(s) underlying the proliferation of heterogeneous cell populations within a solid tumour. To do so, we have developed a model system consisting of NBT-II rat bladder carcinoma cells expressing or not expressing a growth factor. We previously described a community effect in vivo between highly tumourigenic cells expressing the acidic fibroblast growth factor (FGF-1) and their non-expressing NBT-II neighbours. The highly tumourigenic cells do not dominate and the community effect allows poorly tumourigenic NBT-II cells to contribute rapidly to tumour formation [28]. Several approaches have been used to elucidate the community effect. The angiogenic activity of FGF-1 is not responsible for the community effect or for rapid tumour growth [29]; NBT-II cells in which the FGFR signalling pathway has been abolished maintain the potential to cooperate with FGF-1-expressing cells indicating that FGF-1 cannot act as a paracrine or juxtacrine effector [30]. As the cells have to be in close contact, among other possibilities, we investigated whether the two different tumour cell populations are coupled through gap junctions and thus, whether they could share second messengers. In this report we show that (i) both carcinoma NBT-II cells expressing and those not expressing FGF-1 are able to be coupled and to communicate in vitro; in coculture the FGF-1-expressing cells and the non-producing cells communicate efficiently, (ii) these cells express the gap junction protein Cx43, (iii) overexpression of Cx43 in these cells does not affect their tumourigenic growth properties, and (iv) significant inhibition of GJIC by a connexin 43 anti-sense strategy does not diminish the tumourigenic potential of the cells nor abolish the community effect.

Materials and methods

Cells. NBT-II cells are derived from a chemically induced rat bladder carcinoma [31]. Transfected NBT-II cells expressing FGF-1 (NSF 14 cells) have been reported elsewhere [32]. Cells were cultured in DMEM supplemented with 10% foetal calf serum, L-glutamine, penicillin, and streptomycin (100 U/ml) in an incubator at 37 °C under 5% CO_2.

Antibodies. The antibody against connexin 43 used for immunofluorescence has been described previously [33]. Monoclonal anti-Cx43 from Transduction Laboratory was used for Western blotting; a polyclonal anti-Cx43 antibody (kind gift from B. Nicholson) was used for immunocytochemistry. The anti-vimentin used was a monoclonal antibody purchased from Amersham.

Plasmids and transfection. The expression vectors carrying the sense and anti-sense rat Cx43 DNA sequences were a gift from G. Goldberg. They contain the rat Cx43 cDNA or its anti-sense sequence under the control of the CMV promoter and contain the Neo resistance gene [34].

NBT-II cells (10^6) were seeded in 10 cm diameter dishes 24–48 h before transfection. Cells were transfected with 20 μg of each DNA as reported elsewhere [28]. Clones were selected in 400 μg/ml of medium containing G418 (Gibco BRL). Among the different selected ones, S14Cx43-NBT-II cells overexpressing the rat Cx43 and AS18Cx43-NBT-II cells stably transfected with anti-sense Cx43 cDNA were chosen for further experiments.

GJIC dye-transfer assays. Confluent cultures on 60-mm dishes were used for studies of GJIC. A 5% (w/v) solution of Lucifer Yellow CH (Sigma, MW: 475.2) in 0.33 M lithium chloride was microinjected into individual cells using an Eppendorf microinjector, as previously described [33]. Intracellular Lucifer Yellow was photographed 10 min after microinjection and intercellular transfer was calculated as the average number of fluorescent cells from 10 to 20 injections per experiment.

To assay for GJIC between the two cell types, NBT-II cells and their FGF-1 producing counterparts (NSF14 cells), the NSF14 cells were labelled with fluorescent beads [35] and mixed with NBT-II cells to obtain confluent cultures. GJIC was estimated after microinjection of individual NBT-II cells with Lucifer Yellow. As the two cell types can be distinguished, each could be individually microinjected and the communication with the two cell types around the microinjected cell was evaluated.

Tumourigenicity in nude mice. Groups of six-week-old female nude mice (*nu/nu* Swiss strain, Iffa Credo, Les Oncins, France) were subcutaneously injected in the flank with control NBT-II cells, FGF-1-producing cells (clone NSF14), and NBT-II cells expressing the sense (S14Cx43-NBT-II cells) and anti-sense (AS18Cx43-NBT-II cells) Cx43 cDNAs, a mixture of NSF14 (0.5×10^6 cells) and NBT-II cells (3.0×10^6 cells), or a mixture of NSF14 cells (0.5×10^6 cells) with AS18Cx43-NBT-II cells (3.0×10^6 cells), or S14Cx43-NBT-II cells (3.0×10^6). In all cases a total of 3.5×10^6 cells was injected. Tumour volumes were monitored every two days for the first two weeks and once a week thereafter. After euthanasia, tumours were removed, part of them were trypsinized and maintained in culture in vitro, and other fragments were placed directly into O.C.T. embedding medium (Tissue-Tek), frozen in liquid nitrogen-cooled isopentane, cryosectioned, and stored at −80 °C for further analysis.

Identification of cells present in the mixed tumours. Fragments of tumours obtained after S.C. inoculation of cell mixtures were grown in vitro on glass coverslips following cell dispersion by trypsinization. Cells were labelled for vimentin intermediate filaments and the nuclei were visualized with DAPI. Only the mesenchymal FGF1-NBT-II (NSF14) producing cells are positive for vimentin expression. Therefore, the ratio between the number of cells scoring positive for vimentin and total cells gives an estimate of the proportion of NSF14 cells as compared to the epithelial NBT-II, S14Cx43-NBT-II or AS18Cx43-NBT-II cells.

Northern blots. Total RNA was prepared according to the RNA plus (Quantum Bioprobe) procedure and 15 μg RNA from each cell clone was electrophoresed on denaturating gel, transferred to a Hybond N membrane, and hybridized with the appropriate ^{32}P-labelled probe.

The Cx43 probe was a 1.5-kb rat Cx43 fragment [36]. As an internal control Northern blots were re-hybridized with a GAPDH probe [37].

Immunofluorescence. Confluent or subconfluent cells grown on glass coverslips were fixed in acetone at −20 °C for 5 min incubated with the

first antibody (anti-Cx43, or anti-vimentin) and binding revealed with a Texas Red or FITC-coupled second antibody. Frozen sections were labelled after acetone fixation and with the same antibodies.

Immunoblotting. Total extracts from cell culture (5 dishes) were prepared after lysis with RIPA buffer. Protein concentrations were determined with Protein Assay Reagent (Bio Rad) and 500 μg of each sample was electrophoresed on a 10% acrylamide gel and transferred onto a Immobilon P Membrane (Millipore). Connexins were detected by incubation with their respective antibodies and then with peroxidase coupled anti-rabbit or anti-mouse IgG incubation and the reaction was visualized by ECL (Amersham) detection.

Results

NBT-II and FGF-1-producing NBT-II cells express Cx43 and display functional GJIC

Cell–cell communication via gap junctions was investigated in parental NBT-II cells and in the various derived cell lines.

NSF14 cells are derived from NBT-II cells and constitutively produce fibroblast growth factor-1 (FGF-1); these cells have greater invasive and tumourigenic potential than the parental cells [32]. NSF14 cells are of fibroblastic phenotype and produce vimentin intermediate filaments which are absent from NBT-II parental cells and also from cells transfected with the Cx43 sense (S14Cx43-NBT-II cells) or anti-sense (AS18Cx43-NBT-II cells) sequences which remained of epithelial phenotype (not shown).

Connexin 43 is expressed in NBT-II and NSF14 cells and the protein was detected by Western blot (Figs. 1 and 2; not shown for NSF14). Immunocytolocalizations

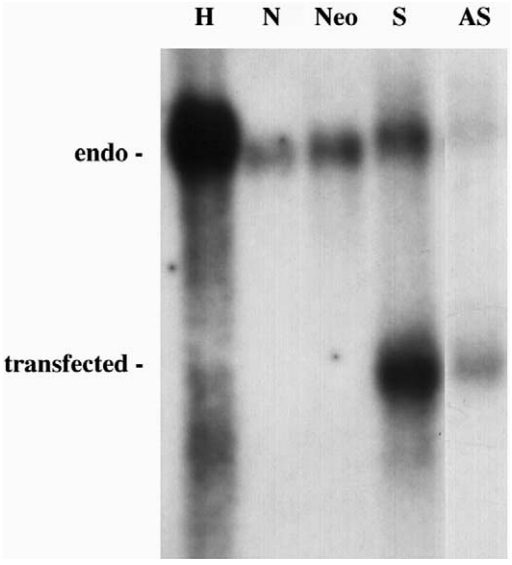

Fig. 1. Northern blot and Cx43 mRNA in control and transfected cells. H: rat heart; N: control NBT-II; Neo: cells transfected with the Neo selective marker; S: S14Cx43-NBT-II cells; AS: AS18Cx43-NBT-II cells. Hybridization with the rat Cx43 probe reveals both endogenous Cx43 expression (endo) and transfected Cx43 cDNA (transfected).

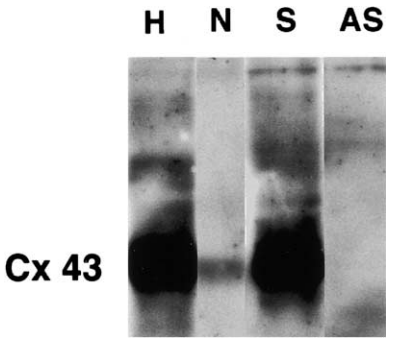

Fig. 2. Western blot analysis for the Cx43 protein. Analysis of protein extracts from rat heart (H), control NBT-II cells (N), S14Cx43-NBT-II cells overexpressing Cx43 (S), and AS18Cx43-NBT-II cells transfected with the antisense Cx43 cDNA (AS).

in confluent cell monolayers with the appropriate antibodies indicate that Cx43 is the major constituent of functional gap junctions in junctional plaques in these cells (Fig. 3a and n).

Cell coupling was determined by injecting Lucifer Yellow into single cells within a confluent monolayer; an average of 34.3 ± 6.6 NBT-II and 21.3 ± 8 NSF14 cells per injected cell were fluorescent and therefore coupled (Fig. 4).

Coculture experiments designed to investigate the putative communication between the two related types

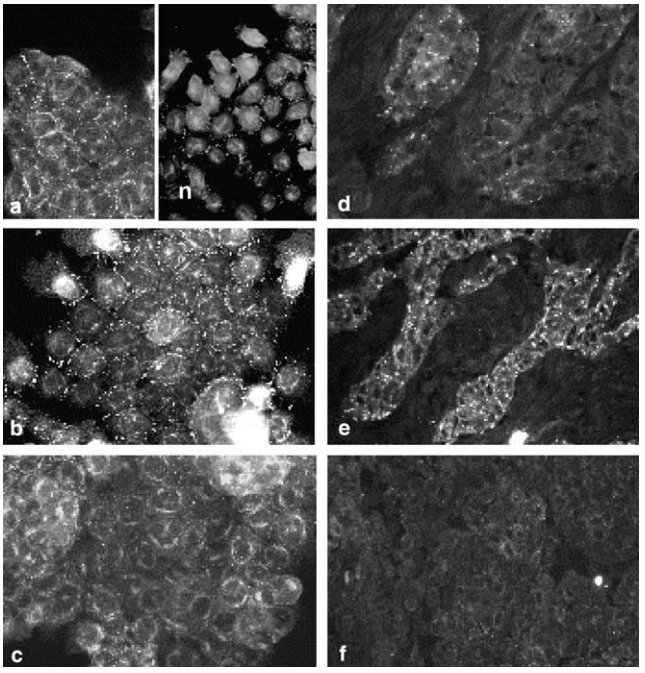

Fig. 3. Immunodetection of Cx43 in cell cultures and in tumour sections. Left panel: immunofluorescent labelling of Cx43 in cells grown on glass coverslips, a: NBT-II cells, n: NSF14 cells, b: S14Cx43-NBT-II cells, c: AS18Cx43-NBT-II cells. Right panel: immunohistochemistry for Cx43 on frozen sections of d: NBT-II tumour, e: S14 Cx43-NBT-II tumour, and f: AS18Cx43-NBT-II tumour.

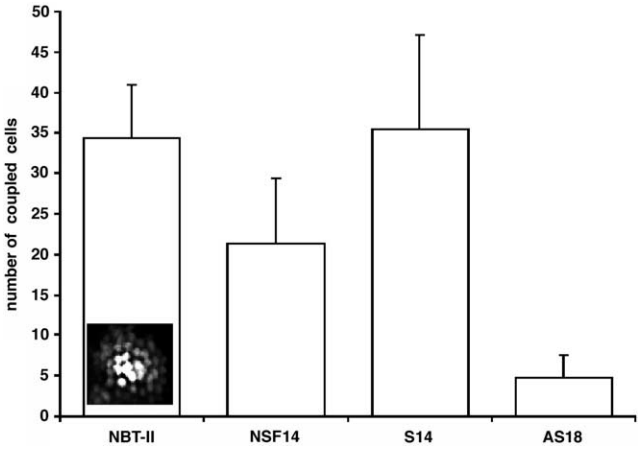

Fig. 4. Cell–cell coupling through GJIC. A single cell within a confluent cell monolayer was injected with Lucifer Yellow: a photo of GJIC for NBT-II cells is presented with the corresponding histogram. Coupled cells were counted and the results for NBT-II cells, NSF14 cells, S14Cx43-NBT-II cells (S14), and AS18Cx43-NBT-II cells (AS18) are means of 10–20 independent microinjections.

of cells (NBT-II and NSF14) indicated that at confluency each cell type is able to communicate which the other. Furthermore, the average number of coupled cells was similar to that obtained for a single cell-type population in culture (data not shown).

Overexpression of connexin 43 is not correlated with increased GJIC

To evaluate the impact of connexin 43 overexpression on intercellular communication, various NBT-II cell clones overexpressing the rat Cx43 were generated by stable transfection. S14Cx43-NBT-II cells, which produced a large amount of the protein as detected by immunoblot, were chosen for further experiments (Fig. 2). These cells are morphologically similar to parental NBT-II cells, i.e, epithelial, but showed dense immunostaining for Cx43 compatible with the protein being localized in plaque junctions (Fig. 3b). However, overexpression of Cx43 in S14Cx43-NBT-II cells was not associated with an increase of the GJIC in vitro. The average cell coupling was 35.5 ± 11.6 cells per injected cell: this value is similar to the value for parental NBT-II cells (34.3 ± 6.6) (Fig. 4).

Generating NBT-II cells incompetent for cell coupling

As the most abundant connexin in NBT-II cells is connexin 43, an anti-sense strategy involving rat anti-sense Cx43 cDNA was used to generate connexin 43-deficient NBT-II cells. Various clones were obtained which produced the anti-sense messenger (Fig. 1) and consequently did not produce the protein. The clone AS18Cx43-NBT-II which did not contain detectable amounts of Cx43 protein (Fig. 2) was used for further experiments. Immunofluorescent staining for Cx43 gave no signal in these transfected cells, consistent with the absence of functional connexin 43 messenger and protein (Fig. 3c). These cells were morphologically similar to parental NBT-II cells and to S14Cx43-NBT-II and had the same epithelial phenotype. Cell coupling was nearly abolished with a mean of 4.8 ± 2.8 per cell versus 34.3 ± 6.6 for parental injected cell (Fig. 4).

Neither overexpression nor absence of expression of Cx43 affects the tumourigenic capacity of NBT-II cells

Subcutaneous injection into nude mice of S14Cx43-NBT-II cell clone gave tumours with kinetics of proliferation similar to those of parental NBT-II cells (Fig. 5). These tumours presented the same pathological carcinoma characteristics as those obtained with untransfected cells. Immunofluorescence analysis of tumour sections with anti Cx43 antibody gave distinct Cx43 signals at cell–cell contact sites further implicating Cx43 in junctional plaques (Fig. 3d–e).

Injection of mice with NBT-II cells deficient for Cx43 expression (AS18Cx43-NBT-II cells) induced tumours that grew with kinetics similar to those of control NBT-II and of CxS14Cx43-NBT-II carcinoma cells. However, tumour section labelling with anti Cx43 confirmed that

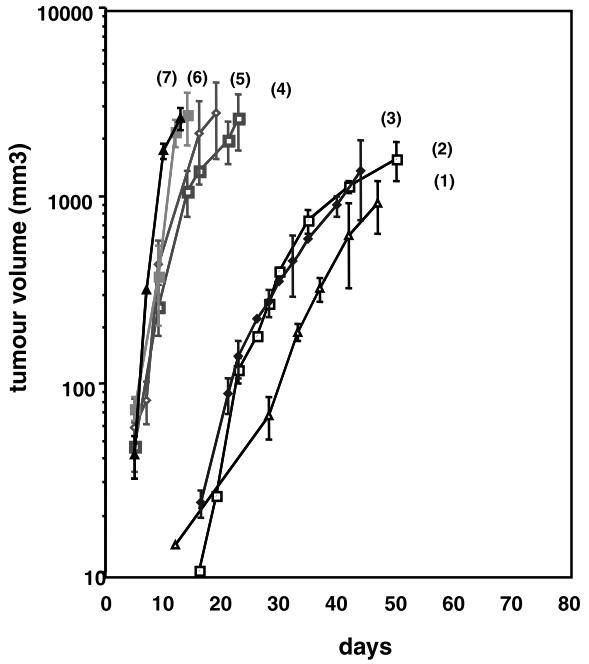

Fig. 5. Tumour proliferation in nude mice. Proliferation of tumours after injection of (1) control NBT-II cells, (7) NSF14 cells (FGF1-NBT-II producing cells), (2) S14Cx43-NBT-II cells (cells transfected with Cx43 sense cDNA), (3) AS18Cx43-NBT-II cells (cells transfected with Cx43 antisense cDNA), and 1:6 mixtures of (6) NSF14:NBT-II cells, (4) NSF14:S14Cx43-NBT-II cells, and (5) NSF14:AS18 Cx43-NBT-II cells. Each point corresponds to the mean of 5–12 tumours and standard errors of the mean are indicated.

in vivo as in vitro these cells did not express Cx43 (Fig. 3f).

The community effect is not dependent on Cx43-mediated GJIC

AS18Cx43-NBT-II carcinoma cells are mostly unable to communicate through gap junctions and were used to test the putative involvement of cell coupling in the community effect. Subcutaneous injection of mixtures of FGF-1-producing cells (NSF14) with NBT-II cells incompetent for cell coupling (AS18Cx43-NBT-II cells) (ratio 1:6) resulted in tumour proliferation similar to that obtained with a mixture of cells competent for cell coupling through gap junctions: either untransfected NBT-II cells or NBT-II cells overexpressing rat Cx43 (S14Cx43-NBT-II cells) (Fig. 5).

Fragments of the different tumours obtained in these experiments were grown in culture after cell dispersion. Immunofluorecence analysis of the vimentin intermediate filaments, which are only present in NSF14-FGF-1-producing cells, showed that all the tumours were heterogeneous. The ratios of the different cell types in these tumours are consistent with the ratios of the cells in the inoculums (1:6) (Fig. 6A and B).

These results indicate that the tumours obtained with the cell mixtures (1:6, cell number:cell number ratio) contained both cell types and were fast growing tumours

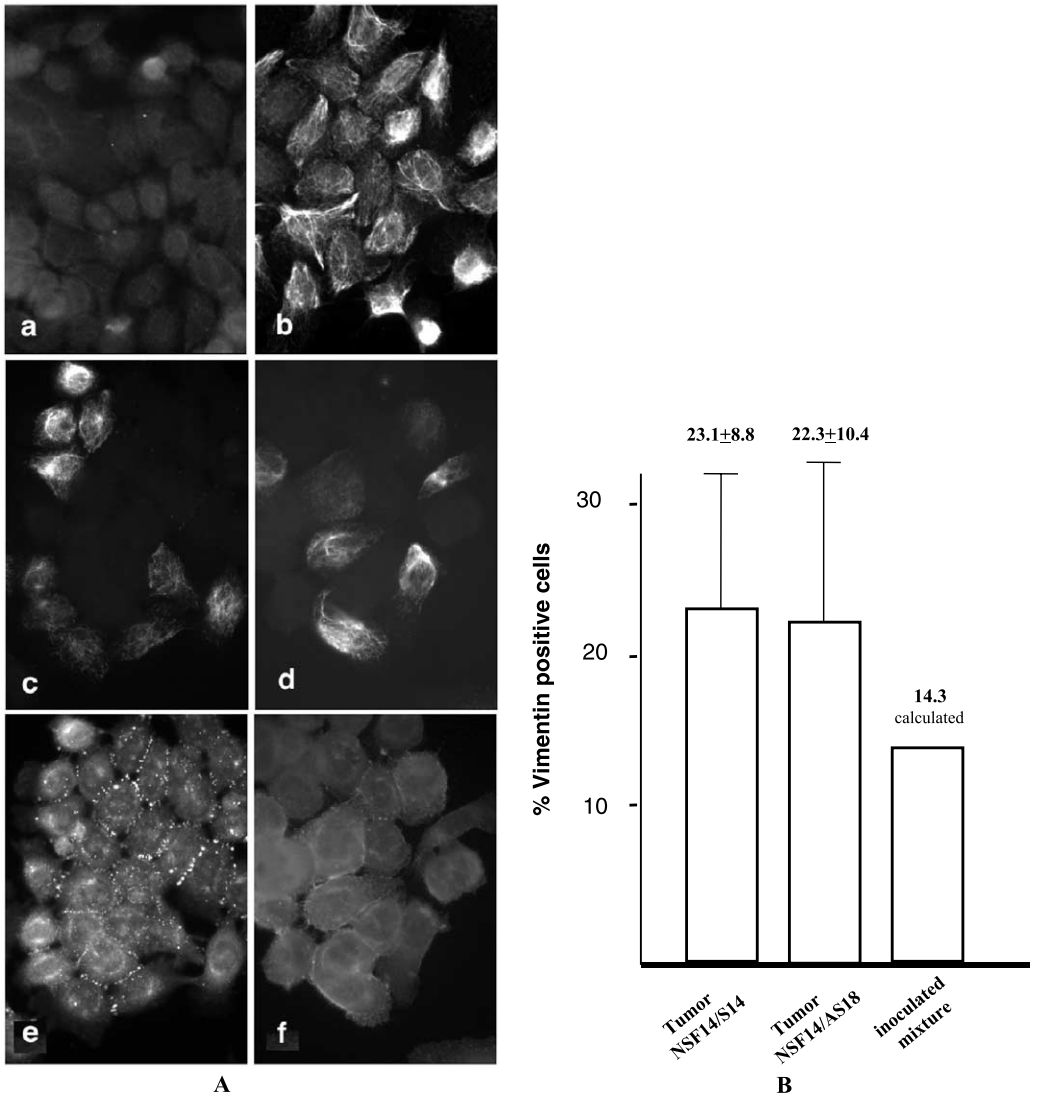

Fig. 6. Cell culture of tumour fragments and cell identification. (A) Fragments of each type of mixed tumour were dispersed and cultured in vitro. Cells grown on coverslips were stained for vimentin (a and b), connexin 43 (e and f), and vimentin (c and d). (a) Control for vimentin in NBT-II tumour cells, (b) control for vimentin in NSF14 tumor cells, (c and e) NSF14:S14Cx43-NBT-II mixed tumour; (d and f) NSF14:AS18Cx43-NBT-II mixed tumour. B: Cells from mixed tumours NSF14:S14Cx43-NBT-II (NSF14/S14) and NSF14:AS18Cx43-NBT-II (NSF14/AS18), grown on coverslips were labelled for vimentin and with DAPI for the nuclei. Means of the ratio of vimentin-positive cells to total cell number were determined for eight fields and compared to calculated ratio in the inoculum.

that proliferated like those obtained with NSF14-FGF-1-producing cells alone or with a mixture of NSF14 and NBT-II cells competent for cell coupling, either NBT-II or S14Cx43-NBT-II cells (Fig. 6A). Thus, there is no evidence that Cx43 is required for the so-called community effect. Consequently this effect seems to be independent of extensive cell coupling through Cx43-containing gap junctions.

Discussion

Findings concerning the role of GJIC in transformed and tumour cells as compared to that in normal cells in adult tissues are contradictory; however, the majority of reports show that GJIC is low in preneoplasic foci, lower in tumours, and possibly even lower or absent in cell lines established from tumours [5]. Endogenous Cx43 expression has been reported to be negatively correlated with neoplastic potential in various neoplasias [6,17] and in bladder cancer cells in particular [15,22]. We have used the NBT-II rat bladder carcinoma cell system to study the impact of cell–cell communication during tumour progression and have shown that the cells are mainly coupled through Cx43-containing gap junctions. NBT-II cells constitutively expressing FGF-1 after transfection are also coupled with Cx43-containing gap junctions and are able to communicate through GJIC with their parental NBT-II cells in vitro.

To assess the effects of cell–cell communication through GJIC during tumour progression, we generated tumour cells incompetent for cell coupling. The first strategy we tried was to transfect NBT-II cells permanently with a dominant-negative Cx32/Cx43 hybrid construct. This construct has been reported to be effective in blocking intercellular communication in *Xenopus* oocytes [38]. Its dominant-negative effect is also effective in endothelial cell-to-cell coupling and subsequently in their wound repair potentialities [44]. Transfected NBT-II cells expressing this construct were however competent for dye transfer indicating that hybrid connexin Cx32/Cx43 is not dominant negative in our system (data not shown).

The anti-sense strategy involving constitutive expression of anti-sense cDNA for rat Cx43 was effective. Two of the clones obtained in which production of the endogenous Cx43 protein was inhibited were defective for coupling as assessed for dye transfer in vitro. These results are in agreement with those of Golberg and collaborators showing that rat-1 fibroblasts transfected with the Cx43 anti-sense construct have a significant decrease in GJIC [34]. Although the in vitro data indicate a loss of GJIC in the AS18Cx43-NBT-II cells as measured by dye transfer in monolayers, this might not accurately reflect the extent of coupling in the tumours. Nevertheless, according to previous work [39] there is good correlation between in vitro coupling of HeLa cells expressing connexins in and their overall in vivo coupling assessed with Lucifer Yellow.

Inoculation of nude mice with these carcinoma AS18Cx43-NBT-II cells deficient for cell coupling gives rise to subcutaneous carcinomas, which developed with growth kinetics similar to those of parental coupled carcinoma cells. The near-elimination of GJIC was not associated with either increased tumourigenic potential or increased growth potential in vivo. Thus, loss of cell coupling in this tumour model does not correlate with an increase in the tumourigenic potential. Unexpectedly, in the NBT-II cell system, overexpression of Cx43 and increase in Cx43-containing junctional plaques was not associated with an increase in cell coupling. The S14Cx43-NBT-II cells that overexpressed rat Cx43 behaved like parental NBT-II cells both in vitro and in vivo for all criteria tested: they had the same epithelial morphology and the same tumourigenic properties. These results indicate that coupling through the endogenous Cx43-containing gaps is not increased when Cx43 is overexpressed after transfection; GJIC was not increased in proportion to the amount of Cx43 protein expressed and localized in the cell membrane. In NBT-II cells, overexpression of Cx43 is not correlated with a tumour suppressive activity in contrast to previous reports in different systems.

The role of gap junctions in bladder tumourigenesis has been investigated and Cx43 has been considered to act as a suppressor gene in certain circumstances. Expression of Cx43 and subsequent restoration of GJIC has been shown to reverse the malignant phenotype in vitro [18]. Overexpression of Cx43 induces a phenotypic reversion towards a less aggressive phenotype in human glioblastoma cells [10]. Similarly there is suppression of tumourigenicity of human lung carcinoma cells after transfection with Cx43 [11,20]. Although there is considerable evidence for a role of GJIC in tumour suppression [40], experiments in genetically engineered mice provide only weak evidence for the direct involvement of gap junctions in growth- and tumour suppression [41]. However, a recent study using conditional expression of Cx43 in HeLa cells reported that Cx43 expression attenuates the tumourigenicity of the cells in immunodeficient mice [42].

Cx43 mutants unable to be targeted to the membrane but able to interact with endogenous connexin have been studied by Krutovskikh et al. [43]. They suggested that connexins influence tumour growth by mechanisms in addition to and distinct from cell–cell coupling: connexins on the cell surface and in the cytoplasm could have different signalling activities, which contribute differently to growth regulation.

The community effect in tumour behaviour was first reported in our laboratory with the carcinoma NBT-II

system. We have demonstrated that even a small subpopulation of NBT-II carcinoma cells that expressed FGF-1 can confer an increased tumourigenic potential to coinoculated untransfected cells [28]. We have demonstrated that direct FGF1/FGFR signalling between the two cell populations is not involved in the community effect [30]. Here, we show that cell–cell coupling through GJIC cannot account for this effect; tumours arising after subcutaneous injection of nude mice with cell mixtures containing FGF1-producing cells and NBT-II cells almost incompetent for cell coupling, are fast growing heterogeneous tumors which contain both the inoculated cell types in a ratio similar to that of the inocula.

It appears that the community effect is established only when cell populations interact closely and that it depends largely on cell interactions between the populations. This also indicates that both cell types of the same origin have the intrinsic ability to give fast growing tumours but the less tumourigenic cell type has to be converted or stimulated by appropriate signalling.

Acknowledgments

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