Polyamines regulate gap junction communication in connexin 43-expressing cells

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The control of cell–cell communication through gap junctions is thought to be crucial in normal tissue function and during various stages of tumorigenesis. However, few natural regulators of gap junctions have been found. We show here that increasing the activity of ornithine decarboxylase, or adding polyamines to the outside of cells, increases the level of gap junction communication between various epithelial cells. Conversely, reduction of ornithine decarboxylase activity decreases the level of gap junction communication. This regulation is dependent upon the expression of connexin 43 (Cx43 or Cxα1), which is a major connexin expressed in many different cell types, and involves an increase in Cx43 and its cellular re-distribution.

Key words: carcinogenesis, keratinocytes, ornithine decarboxylase.

INTRODUCTION

The control of cell–cell communication through gap junctions is thought to be vital in the development and maintenance of animal tissues. These cell-surface structures allow for the diffusion of low-molecular-mass substances directly between the interior of cells. As such, gap junctions are agents that co-ordinate the electrical and metabolic activities of populations of 'coupled' cells [1,2], and integrate the hormonal response in a tissue by sharing low-molecular-mass intracellular signals [3]. However, little is known about the mechanisms that control the level of gap junction communication between cells, particularly those that increase this form of cell–cell interaction.

Gap junctions in vertebrates are comprised of a family of proteins, the connexins [4]. A connexon, formed from six connexin protomers within one cell membrane, docks end-to-end with a connexon in the apposed cell membrane to form a continuous channel between two cells. These channels cluster into supramolecular domains forming the familiar gap junction. Every connexin has four transmembrane domains and is structured with the N- and C-termini residing in the cytoplasm. A total of 14 connexin genes have so far been identified in the mouse genome. The transmembrane regions show high conservation, and the connexins vary principally in the length and sequence of the C-terminal cytoplasmic domain. It is this domain that is thought to be the site which regulates channel permeability and gating [5]. This variability, together with possible variability in the kind of cytoplasmic solutes which can pass through the channels [6], may account for the large number comprising the connexin family.

Consistent with a role of gap junction communication in normal tissue function is the finding that the level of gap junction communication changes in the diseased state. Most transformed cells show reduced levels of gap junction communication, and those that do retain near normal levels of gap junction communication generally are unable to communicate with the normal cells from which they were derived [7,8]. The transformed phenotype can be reversed by transfecting such cells with connexins, which also restores gap junction communication [9–13]. Moreover, connexin 32 (Cx32 or Cxβ1) null mice have an increased incidence of spontaneous and chemically induced hepatoma [14] (hepatocytes express high levels of Cx32). Whereas mice with a heterologously deleted *Cx43* (*Cxα1*) gene have a similar incidence of chemically induced epidermal skin tumours as wild-type littermates [15], cell lines derived from Cx43 null mice have transformed properties, including spindle morphology and increased growth rates and saturation densities [16]. Expression of Cx43 has also been found to down-regulate cyclins and cyclin-dependent kinases in transformed cells [17]. Cx26 (Cxβ2) has been identified as a class II tumour suppressor in mammary epithelial cells [18]. There is, therefore, a large body of evidence supporting a loss of gap junction communication in later stages of tumour progression. However, the situation may be reversed in earlier stages.

In mammalian epidermis, PMA is a potent tumour-promoting agent causing benign papillomas after initiation. Although many cultured cells respond to PMA by a marked reduction in gap junction communication [19,20], in intact skin PMA increases the level of Cx43 and Cx26, major connexins in the epidermis [21,22]. The level of gap junction communication is not reduced after PMA application and, in fact, there is an increase in the incidence of 'promiscuous' gap junction communication between epidermis and dermis [23]. The high expression of Cx43 and Cx26 mirrors the situation in the hair follicle, where rapid cell proliferation is also associated with extensive gap junction communication [24]. Additionally, in benign epidermal hyper-

Abbreviations used: ODC, ornithine decarboxylase; DFMO, α-difluoromethylornithine; MAP, mitogen-activated protein; MEK1, MAP kinase kinase 1.

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plasia, as in psoriasis, viral warts, or after superficial mechanical damage, connexin expression (especially Cx26) is greatly increased [25]. The question therefore arises as to what could be the link between cell proliferation and gap junction communication/connexin expression. One possibility is an increase in the activity of the enzyme ornithine decarboxylase (ODC), which occurs, for example, in skin upon exposure to PMA [26].

ODC catalyses the first step in the synthesis of polyamines, converting ornithine into putrescine [27]. Polyamines are essential metabolites, and they appear to have diverse functions in many different cellular locations. In addition to exposure to PMA, ODC activity in the epidermis is increased in other hyperplastic situations [28,29]. Overexpression of ODC in the skin also acts as a tumour promoter, negating the requirement of PMA after first initiating with a chemical mutagen [30] or expression of an activated Ras [31,32]. Such papillomas regress when animals are fed an inhibitor of ODC activity in their drinking water [31].

In the present study we explored the possible link between levels of ODC activity, polyamines and gap junction communication. We found that increasing ODC activity led to an increased level of gap junction communication. The ODC-induced effect could be mimicked by addition of polyamines to the culture medium. The polyamine effect on gap junction communication levels is dependent upon expression of Cx43, one of the most widely expressed connexins.

EXPERIMENTAL

Cell culture

SP-1 cells were grown to 60 % confluence and infected with the control virus or the ODC virus [33,34] for 5 h with 4 μg/ml Polybrene. The cells were then washed with PBS and fed with Eagle's Minimal Essential Medium containing 8 % Chelex-treated fetal bovine serum. Selection with Geniticin (G418; 120 μg/ml) was begun 3 days after infection and maintained thereafter at 120 μg/ml. Cells were selected for at least 10 days prior to experimentation. In all cell experiments quantitation of intercellular communication by Lucifer Yellow CH was performed 3 days after the last re-feeding. Where stated, putrescine or spermidine (5 mM) was added 24 h prior to the harvest/microinjection. α-Difluoromethylornithine (DFMO; 200 μM) was added in the fresh medium 3 days prior to the harvest/microinjection. HeLa cells transfected with Cx43 were obtained from H. Yamasaki, International Agency for Cancer Research, Lyon, France [35].

Embryo and primary keratinocyte cultures

Primary human epidermal keratinocytes were isolated from foreskin by digestion at 4 °C with dispase and trypsin, and maintained in serum-free keratinocyte growth medium (Bio-Whittaker, Walkersville, MD, U.S.A.) containing 0.15 mM Ca^{2+} [36]. Mouse epidermal keratinocytes were cultured from skins of individual embryonic day 1 embryos from mating of Cx43+/− × Cx43+/− mice [37] (B6,129-Gja1 < tm1Kdr + / − mice; Jackson Laboratories, Bar Harbor, ME, U.S.A.) as described by Hager et al. [38]. Briefly, tail tissue was taken from each embryo to determine Cx43 genotype by PCR. The skin was then removed from the carcass and epidermis separated by treatment with trypsin in PBS overnight at 4 °C. Epidermal cells were collected and plated in collagen type IV-coated dishes in keratinocyte growth medium containing 0.06 mM Ca^{2+}. Keratinocyte cultures derived from each embryo were kept separate and labelled according to Cx43 genotype. Cultures approaching confluence were used for Lucifer Yellow CH microinjection experiments.

Extract preparation

Cells were washed 3 × with PBS and lysed in 10 mM Tris, pH 7.4, 1 % SDS, 1.0 mM Na_2VO_3, 1 mM NaF, and protease inhibitors (1 μg/ml each of aprotinin, pepstatin and leupeptin, and 0.2 mM PMSF). The lysates were then sonicated for 30 s on ice to shear the DNA, followed by centrifugation at 12000 ***g*** for 20 min at 4 °C. Equal amounts of cellular protein from the supernatants were analysed by SDS/PAGE (10 % gels) and transferred to nitrocellulose. Blots were stained briefly in Ponceau S (Sigma) to verify equal loading, and Western blotting was performed using polyclonal antibody (1:1000 dilution) to detect Cx43. The Cx43 polyclonal antibody was kindly provided by Dr E. Rivedal (Institute for Cancer Research, Oslo, Norway). Proteins were visualized using chemiluminescence detection.

Immunofluorescence

SP-1 cells were grown on chamber slides (Lab-Tek; Nalge Nunc, Life Technologies) and fixed in acetone. The cells were then blocked with 20 % normal horse serum and reacted with monoclonal anti-Cx43 antibody (1 μg/ml; Signal Transduction Laboratories, Lexington, KY, U.S.A.) for 2 h. Bound antibodies were detected with a fluorescein-conjugated anti-mouse IgG antiserum.

Measurement of gap junction intercellular communication

Lucifer Yellow (4 % in water; Sigma) was injected by iontophoresis (10 nA, 0.5 s pulses at 1 Hz for 2 min) into cells in confluent areas. At 1 min after each injection (20 injections per dish) cells with detectable Lucifer Yellow fluorescence were counted. A minimum of 20 injections was used in each experiment.

RESULTS

Effect of ODC overexpression on gap junction communication

SP-1 cells were infected with a replication-defective retroviral vector containing the *ODC* gene. The gene encoded for a truncated isoform of ODC which was known to increase the half-life of the enzyme, and a previous study has shown that it led to a marked increase in levels of polyamines inside SP-1 cells, notably putrescine [33,34]. SP-1 cells were derived from mouse epidermal keratinocytes, have an activated *ras* gene and the major connexin expressed was Cx43 (see below). For a control, the same retroviral vector was used without the *ODC* gene, and both viruses contained a neomycin resistance gene allowing for selection of infected cells.

Single control and ODC-infected SP-1 cells were injected with the membrane-impermeable dye Lucifer Yellow by iontophoresis 72 h after last being re-fed when cultures had just reached confluency. ODC-overexpressing cells consistently exhibited much larger dye spreads than control infected cells (Figures 1A–D). In three separate experiments there was a 3–5-fold increase in gap junction communication (Figure 1G). This increase could be completely blocked by addition of DFMO, a specific inhibitor of ODC enzymatic activity (Figures 1E, 1F and

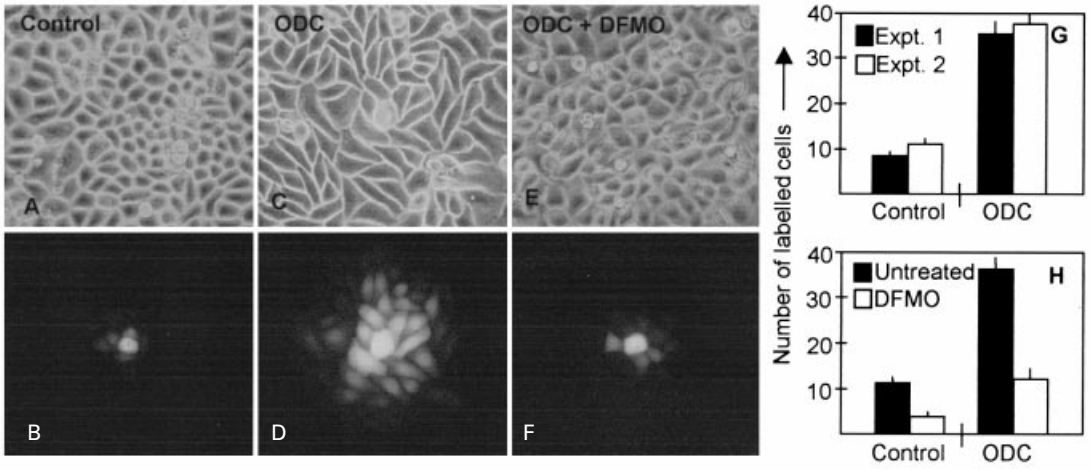

Figure 1 Spread of Lucifer Yellow in control and ODC-infected SP-1 cells in the presence or absence of DFMO

Micrographs show the phase contrast (**A**, **C**, **E**) and corresponding fluorescent images (**B**, **D**, **F**) of SP-1 cells infected with the control virus (**A**, **B**), ODC virus (**C**, **D**), and ODC virus followed by incubation with DFMO (**E**, **F**). (**G**) The mean ± S.E.M. number of cells that the Lucifer Yellow dye has spread to in two separate experiments of SP-1 cells infected with control and ODC virus is shown. (**H**) The mean ± S.E.M. number of cells that the Lucifer Yellow dye has spread to in control and ODC-infected SP-1 cells, in the absence or presence of the ODC inhibitor, DFMO. $P < 0.00005$ for each experiment between control-infected and ODC-infected and DFMO-treated cells.

1H). These results suggested that intracellular levels of polyamines regulated the level of gap junction communication.

Addition of polyamines to the culture medium of SP-1 and other epithelial cells

It might be expected that adding polyamines to the culture medium of SP-1 cells would mimic the effects on gap junction communication observed by elevated levels of ODC activity. An experiment was carried out to examine this possibility. Putrescine was added to the medium of sub-confluent cultures of SP-1 cells for either 6 h or 24 h at concentrations of 1 mM or 5 mM, and the extent of gap junction communication measured. This polyamine was chosen because it was the most prevalent in ODC-infected cells [33,34].

Treatment with 1 mM putrescine for 6 h or 24 h had no effect (control dye spread, 5.1 ± 1.73 cells; 6 h time point, 3.6 ± 1.35 cells; and 24 h time point, 7 ± 3.02 cells). Similarly, 5 mM putrescine for 6 h only marginally raised the extent of gap junction communication (8.12 ± 4.99 cells). However, in the two experiments shown (Figure 2), putrescine treatment for 24 h at a concentration of 5 mM increased gap junction communication by an average of 2.5-fold. Addition of spermidine at a con-

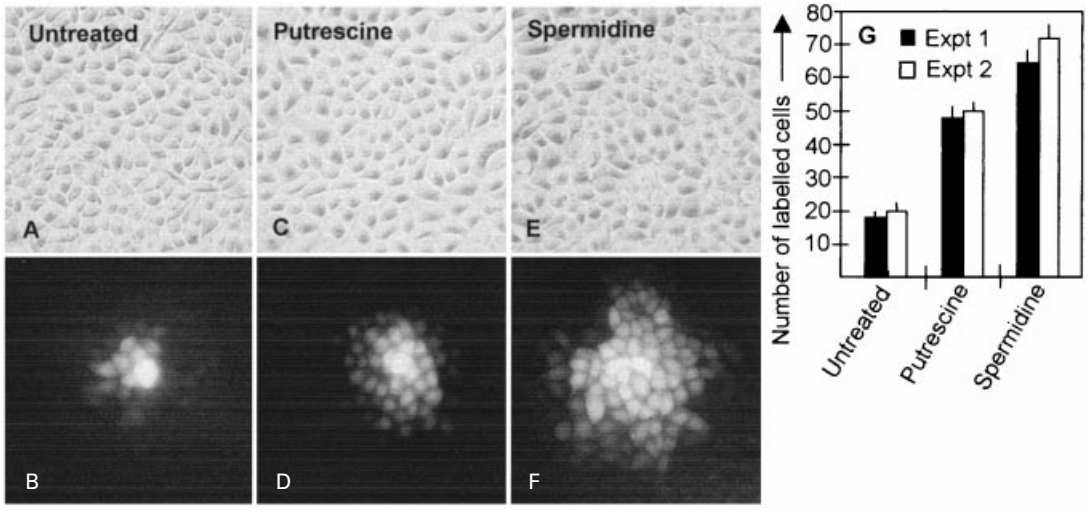

Figure 2 Spread of Lucifer Yellow in SP-1 cells cultured in the presence of the polyamines, putrescine and spermidine

SP-1 cells were cultured in the absence (**A**, **B**) or presence of putrescine (**C**, **D**) or spermidine (**E**, **F**) for 24 h before examining the gap junctional communication by the spread of Lucifer Yellow. (**A**, **C**, **E**) Phase contrast image, (**B**, **D**, **F**) the corresponding fluorescence image. (**G**) The mean ± S.E.M. number of cells that Lucifer Yellow has spread to for two separate experiments. $P < 1 \times 10^{-8}$ for each experiment between untreated and polyamine-treated cells.

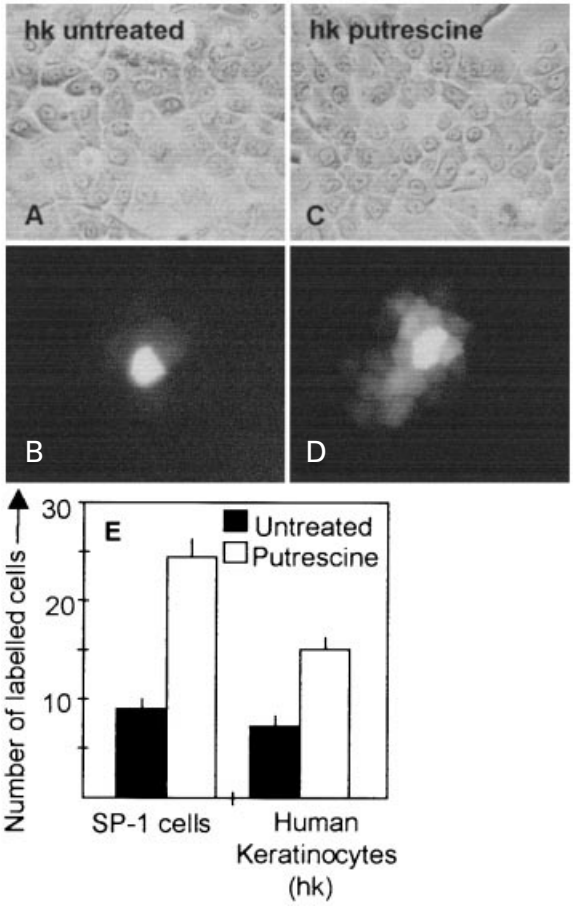

Figure 3 Spread of Lucifer Yellow in cultures of SP-1 cells, Balb mouse keratinocytes and primary human keratinocytes in the presence or absence of putrescine

Micrographs show the spread of Lucifer Yellow between primary human keratinocytes (hk) in control cultures (**A**, **B**) and cultures treated for 24 h with putrescine (**C**, **D**). (**A**, **C**) Phase contrast image, (**B**, **D**) the corresponding fluorescence image. The histogram shows the mean ± S.E.M. number of cells that Lucifer Yellow has spread to for the two cell types. $P < 0.000001$ between untreated and putrescine-treated cells.

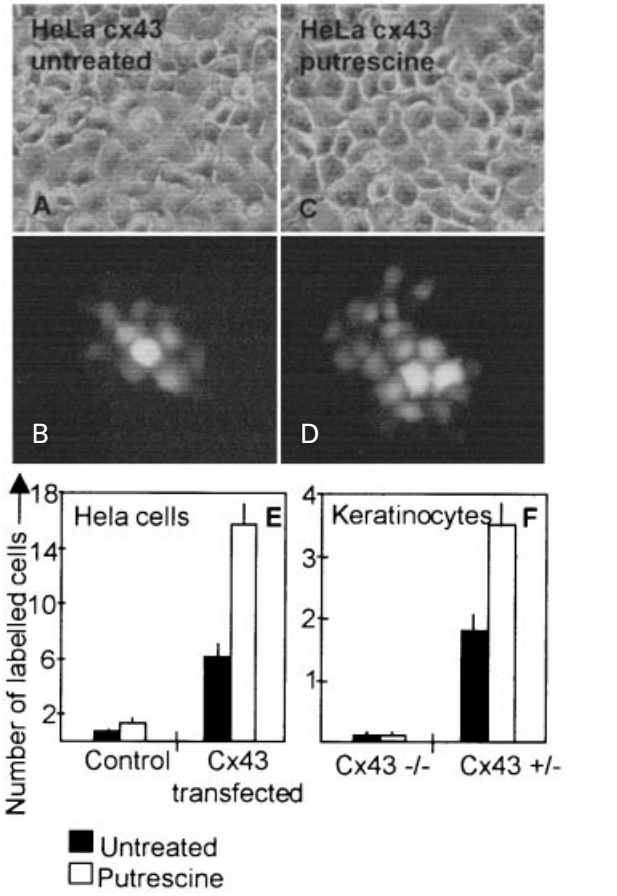

Figure 4 Effects of putrescine on HeLa cells expressing Cx43 and primary keratinocytes from Cx43 null mouse

Micrographs show HeLa cells transfected with Cx43 in untreated cultures (**A**, **B**) and cultures treated for 24 h with putrescine (**C**, **D**). (**A**, **C**) show the phase contrast image, (**B**, **D**) the corresponding fluorescence image. The histograms show the mean ± S.E.M. number of cells Lucifer Yellow has spread to for the three cell types. There is no statistical significance between control (non-Cx-transfected HeLa cells) in untreated and putrescine-treated cultures, or between untreated and putrescine-treated keratinocyte cultures from Cx43 null neonates (Cx43 − / −). $P < 0.001$ between untreated and putrescine-treated keratinocytes from heterozygous neonates, Cx43 + / −.

centration of 5 mM for 24 h increased gap junction communication by over 3.5-fold (Figures 2F and 2G). As expected, addition of putrescine to the culture medium of either ODC or control-infected SP-1 cells counteracted the inhibition of gap junction communication by DFMO (see Figure 6).

Putrescine was added to the culture medium of a variety of other cells to determine if the increase of gap junction communication was a widespread phenomenon (Figure 3). These cells included HeLa (human cervical carcinoma), A9 (*hprt*$^-$ variant of the mouse L929 cell line) and normal primary human keratinocytes. These cells were chosen as representatives of various stages of the transformation process, as well as having a varying range of abilities of gap junction communication.

Primary human keratinocytes increased gap junction communication 2-fold after putrescine treatment. There was no change in HeLa and A9 cell lines (Figure 4E; results not shown for A9 cells). These results showed that the effects of polyamines were not limited to SP-1 cells, and that gap junction communication can be increased among non-tumorigenic and primary epidermal cells. However, the addition of putrescine to poorly or non-communicating cell lines did not induce gap junction communication, indicating a missing component.

Putrescine affects Cx43 expression

Previous studies have shown that A9 and HeLa cells were deficient in known connexins [35], whereas the cell types which responded to putrescine were known to express Cx43 (Figure 5 and results not shown). The apparent requirement for Cx43 was examined using two genetic approaches: in one approach, Cx43-expressing HeLa cells were used [35]; and in the other, primary keratinocytes were established from newly born pups from crosses of Cx43 heterozygote mice [37].

HeLa cells expressing Cx43 have been shown by others [5] to increase gap junction communication. Measurement of gap junction communication showed dye spreads to five or six other cells, but treatment with putrescine caused a 3-fold increase in the spread of the dye (Figure 4). In contrast, primary keratinocytes established from Cx43 null mice had lost gap junction

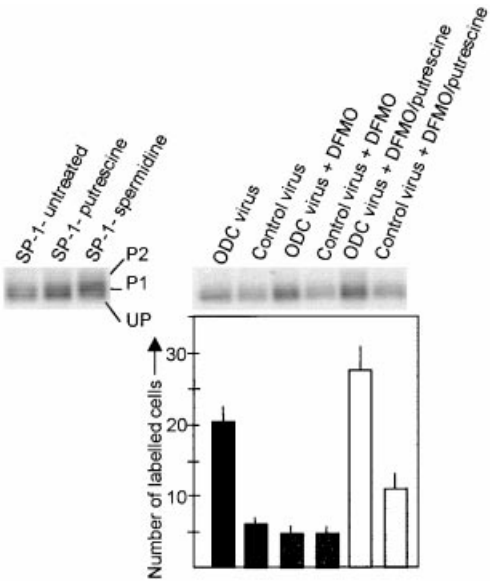

Figure 5 Cx43 immunoblots from SP-1 cells and Balb mouse keratinocyte cells

Left panel: immunoblots for Cx43 of untreated and polyamine-treated cultures of SP-1 cells. Marked are the presumptive unphosphorylated (UP) and two phosphorylated isoforms (P1 and P2). Right panel: immunoblots for Cx43 from SP-1 cells after infection with control or ODC virus and after treatment with DFMO or putrescine, or both together. Bottom panel: results from a companion experiment of Lucifer Yellow spread. The mean ± S.E.M. number of cells Lucifer Yellow has spread to is shown.

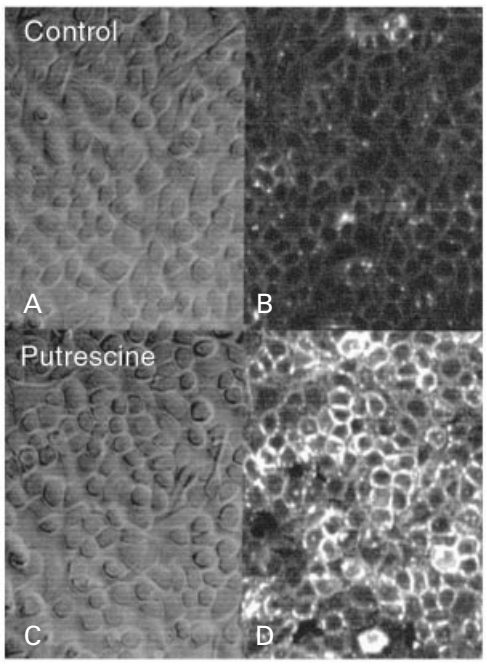

Figure 6 Cx43 immunofluorescence in untreated and putrescine-treated cultures of SP-1 cells

(**A**, **B**) Phase contrast and corresponding fluorescence images of untreated SP-1 cell cultures. (**C**, **D**) Phase contrast and corresponding fluorescence images of putrescine-treated SP-1 cell cultures.

communication, and showed no increase in gap junction communication after addition of putrescine. However, primary keratinocytes established from heterozygous littermates had a low, but consistent, level of gap junction communication, which could be increased 2-fold by treatment with putrescine.

Northern-blot analysis showed no increase in Cx43 mRNA expression in ODC-infected SP-1 cells or putrescine-treated SP-1 cells (results not shown). However, immunoblotting (Figure 5) showed a variable increase in the amount of Cx43 in ODC-infected and putrescine-treated SP-1 cells. Exposure to spermidine also resulted in the amount of slower migrating forms of Cx43 consistent with phosphorylation. Somewhat unexpected was the finding that the amount of Cx43 did not necessarily follow changes in gap junction communication. For example, ODC-infected SP-1 cells treated with the inhibitor DFMO caused an increase in Cx43 but no change in gap junction communication.

Immunofluorescence of Cx43 showed a marked increase in staining at the cell surface of SP-1 cells after either ODC infection or putrescine treatment (Figures 6A–D; results shown only for putrescine-treated SP-1 cells). However, staining was not uniform, and there appeared to be groups of cells which showed little staining above control cells.

Polyamine activation by a mitogen-activated protein (MAP) kinase-independent pathway

SP-1 cells have a mutant form of Ras leading to activation of the Ras/extracellular-signal-regulated protein kinase ('ERK') signalling pathway [39]. As polyamines were known to activate kinase-dependent pathways [40], it could be that the increased gap junction communication observed in these cells by elevation of polyamines was due to interference of this pathway. This possibility was examined with an inhibitor of MAP kinase kinase 1 (MEK1), U0126, which is responsible for activation of the MAP kinase known to be a required event in this pathway [41].

Sub-confluent cultures were incubated with U0126 (50 μM), or spermidine (5 mM) or with both (Figure 7). The concentration of U0126 used was known to inactivate MAP kinase activation in keratinocytes [41], and spermidine was chosen to maximize the increase in gap junction communication. Incubation with U0126

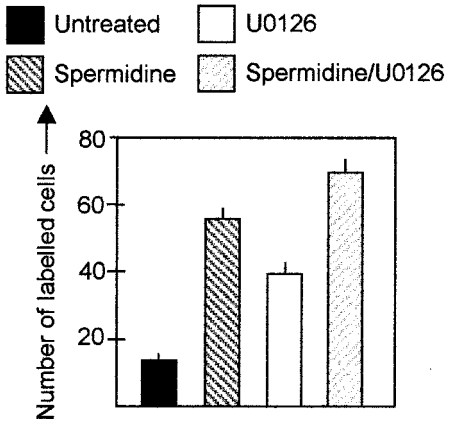

Figure 7 Effect of U0126 and spermidine on gap junction communication in SP-1 cells added separately or together

The mean ± S.E.M. number of cells Lucifer Yellow has spread to after the various treatments is shown. $P < 0.5 \times 10^{-9}$ between untreated and treated cells; $P < 0.005$ between spermidine and spermidine/U0126-treated cells.

alone caused a 2.5-fold increase of gap junction communication, while spermidine resulted in a 3.5-fold increase. However, in combination, there was a further increase to over 4.5-fold, showing an additive effect ($P < 0.005$ between spermidine and spermidine + U0126).

DISCUSSION

Gap junctions are essential channels of cell–cell communication in multicellular animals. They provide an integrated transfer network for the movement of inorganic ions and low-molecular-mass cytoplasmic solutes within tissues, thereby co-ordinating the activity of cells in tissues. It would, therefore, be expected that there might be mechanisms to regulate the extent of gap junction communication. To date, however, few naturally occurring molecules have been found which can modulate gap junction communication, and those that do tend to decrease the extent of gap junction communication, e.g. oleamide [42].

The data presented in the present study lead us to conclude that the activity of ODC and consequent changes in polyamine synthesis determine the extent of gap junction communication in at least some cell types. Our conclusion is based on a previous study in SP-1 cells [33], which has shown that after infection with the ODC virus, there is a marked elevation in cellular polyamine levels, notably putrescine. Although we have not measured cellular polyamine levels after adding putrescine and spermidine to the outside of cells, it seems likely that this too would lead to elevated intracellular levels of polyamines. Supporting this is the finding that many cells in culture accumulate fluorescent-tagged polyamines [43]. Our conclusion that polyamines can markedly increase gap junction communication is, therefore, one of the first instances, alongside cAMP [44], where a family of naturally occurring metabolites can have long-lasting effects on the regulation of gap junction communication.

The observed stimulation of gap junction communication is dependent upon expression of connexins, notably Cx43. The mechanism appears to involve an increase in total cellular Cx43 and its appearance at the cell surface. This is consistent with an *in vivo* study [22] showing that increased ODC activity by topical application of PMA is associated with increased Cx43 and Cx26 expression in mouse epidermis. The redistribution is consistent with a recent study [45] showing that the size of the gap junction domain is important. The size of the junction plaques of Cx43 has recently been shown to be important, and accumulation into large domains is needed to establish gap junction communication. The immunocytochemical analysis after treatment with polyamines is consistent with this finding. An analogous situation occurs in a mouse mammary tumour cell line treated with cAMP, which causes a marked increase in gap junction communication and the phosphorylation of Cx43, as well as the redistribution of Cx43 to gap junction domains [44]. In the case of polyamines, however, phosphorylation of Cx43 appears not to be necessary for stimulation of gap junction communication, although addition of spermidine is accompanied by the appearance of phosphorylated forms of Cx43.

The effect of polyamines appears to be independent of the Ras/MAP kinase-signalling pathway. In SP-1 cells the activation of this pathway is associated with a loss in the extent of gap junction communication. Thus the addition of the MEK1 inhibitor, U0162, results in an increase in gap junction communication. However, down-regulation of this signalling pathway does not abrogate the response to spermidine. In the examples we have used, the response to increased polyamines is not confined to normal cells but is retained by transformed cells, such as SP-1 cells and HeLa cells, suggesting the mechanism is not cell-type specific and is retained.

What is the significance of regulation of gap junction communication by polyamines? One possibility is that it is related to the growth rate of tissues. Elevated ODC activity occurs in many situations where growth rate is increased, and this is thought to be essential to maintain such growth. For example, ODC activity is increased after UV irradiation of the epidermis [46] and in the growing hair follicle [28]. It is also elevated in hyperplastic diseased states such as psoriasis and papillomas [29,47]. Indeed, high expression of ODC in the epidermis circumvents the need for exposure to tumour promoters such as PMA to form papillomas, and such papillomas regress when ODC activity is inhibited by DFMO [31,32]. It may be beneficial to increase the level of gap junction communication to co-ordinate growth activities and to allow for the intercellular movement of metabolites to maintain the increased growth rate.

It is generally thought [2] that gap junction communication is decreased at tumour promotion, and that this decrease may be essential for the dysregulation of growth control. For example, PMA reduces gap junction communication in many different cell types, as does transfection with papillomaviruses or papillomaviral E5-transforming gene [48,49]. Rous sarcoma virus oncoprotein vSrc60 is also known to reduce gap junction communication and appears to operate via tyrosine phosphorylation of Cx43 [50]. However, any decrease during promotion, while possibly essential to the transformation process, may be a transient feature of early tumours such as papillomas. The pressures to increase gap junction communication for the dispersal of nutrients may outweigh any advantage of repressing it. Ras activation of MAP kinase-signalling pathways in SP-1 cells appears to reduce gap junction communication. However the stimulation of gap junction communication by polyamines appears to be independent of this signalling pathway. This leads to the possibility that papillomas which carry an activated Ras will still increase gap junction communication in response to elevated ODC activity. Our results suggest that regulation of gap junction communication could be a major function of polyamines in mammalian tissues. Further studies are needed to elucidate the details of the signalling mechanisms involved.

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