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Research Article

Retinoic acid modulates gap junctional intercellular communication in hepatocytes and hepatoma cells

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Abstract. Gap junctional communication permits the direct exchange of small molecules and ions and has been implicated in tissue homeostasis/metabolite exchange. The lack of gap junctional intercellular communication (GJIC) plays important roles in the promotion and progression of carcinogenesis. In the present study, we demonstrate that treatment of human hepatoma Hep G2 cells with retinoic acid (RA) results in increased amounts and phosphorylation of connexins, their stabilisation in plasma membrane plaques and enhanced GJIC. In cultured fetal hepatocytes, which represent a non-transformed, proliferating and incompletely differentiated liver system, the effects of RA are limited to the establishment of connexin in areas of cell-cell contact and the improvement of GJIC. This suggests that modulation of cell-cell channel communication by RA occurs differently in these two experimental models: while RA is able to revert cell transformation in Hep G2 cells, in fetal hepatocytes it may induce the expression of a more differentiated phenotype.

Key words. Retinoic acid; connexin 32; connexin 43; hepatocyte.

Gap junctions present in epithelial cells provide the only route for direct exchange of cytoplasmic components with a molecular weight less than about 1000 Da [1]. Gap junctional intercellular communication (GJIC) has long been postulated to play an important role in maintaining homeostasis and in the control of cell growth [2]; in addition, loss of GJIC has been considered to cause abnormal development and tumour formation [3]. Accumulating evidence indicates that the loss of GJIC in transformed malignant cells is closely correlated with transcriptional down-regulation of certain tissue-specific connexin (Cx) genes that, in turn, when up-regulated, may inhibit cell growth in some tumours [4]. In studies using in vitro cell systems, GJIC was inhibited by agents that promote tumours; moreover, transfection of neoplastic cells with Cx genes allows the reconstitution of normal conditions. Hence, a tumour suppressor role for Cx genes has been postulated [5].

GJIC can be regulated at different points, such as transcription, mRNA stabilisation, translation and post-translation processing. Second messengers of signal transduction, such as Ca^{2+}, cAMP and inositol triphosphate, are also involved in the regulation of gap junctions [6].

Gap junctions consist of aggregated membrane channels, each channel being composed of two connexons, one from each of two juxtaposed cells. Each connexon consists of six Cx molecules, and each gap junction contains several hundred connexons [7]. At least 15 different Cx genes have been cloned from rodents and the expression of various combinations of Cxs has been reported in different tissues [8, 9]. In the liver, GJIC involves at least three different connexins, Cx32, Cx26 and Cx43, depending on the cell type or cell position in the lobule [10].

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In vivo and in vitro, normal hepatocytes mainly express Cx32 (about 90% of liver gap junction proteins) and Cx26, whereas hepatoma cell lines express essentially Cx43. The liver, both in vivo (i.e. during regeneration or carcinogenesis progression) and in vitro (i.e. following dissociation and culture of cells, either quiescent or induced to proliferate), represents a good model to study the dynamic disassembling and reconstitution of gap junctions and to investigate the relationship between GJIC and cell proliferation [11].

Many substances, such as hormones, growth factors and drugs, positively or negatively affect GJIC; we focused our attention on retinoids because of their supposed roles in tumour prevention and therapy as well as in the improvement of GJIC, as reported in some cell systems [12].

In previous reports, we demonstrated the antiproliferative, differentiative and/or apoptotic effects of retinoic acid (RA) on rat hepatocytes [13, 14] or Hep G2 cells [15], by analysing morphological and physiological parameters. The aim of the present research was to investigate whether the previously observed effects could be somehow correlated with increased intercellular communication through the modulation of gap junction components.

Materials and methods

Animals and cell cultures

Pregnant Wistar rats (20th–21st day of gestation) were kept on a standard diet ad libitum and with free access to tap water, according to the Ethical Principles of Laboratory Animal Care. Before surgery, animals were anaesthetised intraperitoneally with Farmotal (Farmitalia, Milan, Italy), 10 mg/100 g body weight. Isolated hepatocytes were obtained from fetal livers with a non-perfusive enzymatic method, as described elsewhere [16]. Cell viability was assessed by the Trypan blue exclusion test and was not less than 95% for each preparation. Cells were then suspended in RPMI 1640 medium, supplemented with 10% fetal calf serum (Euroclone, UK), 2 mM L-glutamine, 2.5 µg/ml amphotericin B, 10^{-7} M dexametasone, 50 U/ml penicillin, 0.05 mg/ml streptomycin and 100 µg/ml gentamicin, and plated on 60-mm plastic dishes (Becton Dickinson Labware), coated with type I collagen (Sigma, St Louis, MO), at a density of 2×10^6 cells/dish. The medium was changed 3–4 h after plating and then every 24 h. Twenty-four hours after plating was considered as time 0; at this time, serum-free conditions were used and 5 µM all-trans RA (Sigma) was added.

The human Hep G2 cells (American Type Culture Collection, Rockville, MD) were suspended in the same medium used for fetal hepatocytes, plated on 60-mm plastic dishes at 10^5 cells/dish, and cultured for 12 days in the absence or presence of 5 µM RA; the medium was changed every 2–3 days. This RA concentration was chosen after a series of preliminary experiments because it is not cytotoxic but produces an adequate cell response.

Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and the experiments were carried out at 48 h for fetal hepatocytes and at 2, 7 and 12 days for Hep G2 cells. These different intervals of RA treatment were chosen on the basis of previous results obtained with these experimental models [13–15]. In particular, while rat primary hepatocytes are more sensitive to RA and allow us to assess early cell responses, Hep G2 cells respond later to RA treatment and, therefore, are more suitable for studying the long-term effects of RA stimulation.

For all experiments, RA was used from a 5 mM stock dissolved in absolute ethanol; dishes without RA were considered controls. Possible ethanol effects were preliminarily excluded using additional dishes containing the corresponding amount of this vehicle alone.

Western blot

For Western blot analysis, hepatocytes and Hep G2 cells were solubilised in 20 mM Tris-HCl buffer containing 50 mM NaCl, 10% glycerol, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 2% SDS, 2 mM sodium vanadate, 16 µg/ml aprotinin, 10 µg/ml leupeptin, 16 µg/ml pepstatin and 1 mM phenylmethylsulphonyl fluoride (PMSF) (Sigma). The suspension was centrifuged at 12,000 g for 30 min and proteins from the supernatant were submitted to 12.5% SDS-polyacrylamide gel (70 µg of cell proteins per lane) electrophoresis according to Laemmli [17]. Proteins were transferred to nitrocellulose sheets (3 h at 20°C) according to procedures described in detail by Towbin et al. [18].

For phosphoserine detection, samples were immunoprecipitated for better detection of the signal. For immunoprecipitation, cells were solubilised in PBS containing 1 mM calcium cloride, 1 mM magnesium chloride, 1 mM PMSF, 1% Triton X-100, 2 mM sodium orthovanadate, 20 µg/ml pepstatin, 20 µg/ml leupeptin and 15 µg/ml aprotinin (Sigma). Samples were centrifuged at 12,000 g for 30 min and aliquots from supernatants, containing 0.5 mg of protein, were pre-cleared with 25 µl of protein G-Sepharose '4 fast flow' (Pharmacia Biotech, Uppsala, Sweden) for 2 h at 4°C, followed by removal of protein G-Sepharose beads by centrifugation (12,000 g for 3 min at 4°C). Lysate was then immunoprecipitated overnight at 4°C with 25 µl of protein G-Sepharose and 4 µg of anti-Cx32 or anti-Cx43 (Zymed Laboratories, San Francisco, CA). The beads bearing the immunoprecipitates were collected by centrifugation and washed with lysis buffer. Proteins were eluted and processed for SDS-PAGE and Western blot as described above.

For immunoblot analysis, the nitrocellulose sheets were incubated (90 min at room temperature) with rabbit anti-Cx32, anti-Cx43 or rabbit anti-phosphoserine antibodies

(Zymed), diluted 1:300, 1:200 or 1:100, respectively in TBS (50 mM pH 7.5 Tris-HCl, 150 mM NaCl), containing 1% non-fat milk powder. Non-specific binding of the membranes had been previously blocked with 5% non-fat milk powder in TBS 0.1% Tween-20 at 4°C overnight. Following incubation with an alkaline phosphatase (AP)-conjugated secondary antibody, protein-antibody complexes were visualised with the BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate/4-nitroblue tetrazolium chloride) system (BioRad). Control blots were set by omitting the primary antibody. Positive bands were then scanned in a densitometer and photographed.

Immunofluorescence

Fetal hepatocytes and Hep G2 cells, grown on coverslips, were fixed in 3.7% paraformaldehyde for 10 min at 4°C, rinsed with 0.2% glycine, permeabilised with 0.05% Triton X-100 for 8 min, and incubated for 1 h at 37°C, with either rabbit anti-Cx32 or rabbit anti-Cx43 (Zymed Laboratories) diluted 1:30 and 1:50, respectively, in PBS 1% BSA. After PBS washing, the cells were incubated for 1 h in Cy3-conjugate goat anti-rabbit IgG (Zymed Laboratories) diluted 1:50 in PBS containing 10% normal goat serum. Negative control slides were performed by exposing cells under similar conditions, while omitting the primary antibody. After appropriate rinsing, the coverslips were mounted on slides in an aqueous medium and the cells were examined under an epifluorescence microscope (Axioplan 2, Zeiss).

Quantitative analysis was attempted on digitised images by counting the number of positive fluorescent spots present on the surface of treated and control cells, using the program KS300 Kontron. Images from three different experiments were analysed. For each experiment, at least three microscopic fields were examined for control or RA-treated cells. Data are expressed as the mean ± SD of positive spots per cell.

Scrape-loading and dye transfer method

Confluent cells grown in monolayers on coverslips were scrape-loaded at room temperature using forceps and incubated for 2 min with a 0.05% solution in PBS (non-cytotoxic concentration) of Lucifer yellow (MW 457.2 Da; Sigma) and rhodamine dextran (MW 10,000 Da; Sigma) following the method described elsewhere [19]. After incubation, the coverslips were rinsed in PBS to remove detached cells and background fluorescence, mounted on slides, and observed under a Zeiss epifluorescence microscope. Negative control coverslips were performed by exposing the cells to the dye mixture under similar conditions but without scraping.

Results

In the first series of experiments we determined the protein levels of Cx32 and Cx43 in Hep G2 cells and of Cx32 in fetal hepatocytes by Western blotting. As shown in figure 1 A, Hep G2 cells displayed no appreciable change in Cx32 concentration at any interval after RA treatment considered, while Cx43 (fig. 1 B), which is characteristic of hepatoma cells, increased on average by 50% at 7 days and by 60% at 12 days. Only a small amount was detectable after 2 days, in both control and RA-treated cells.

Evidence is mounting that the state of phosphorylation of Cxs and, in particular, their phosphorylation on serine residues, plays a role in Cx assembly, in their insertion in plasma membrane plaques and in the regulation of channel gating and functioning [7]. We, therefore, next evaluated the level of Cx phosphorylation using a specific phosphoserine antibody. The results of these studies showed a high increase (about 90%) in the Cx32 phos-

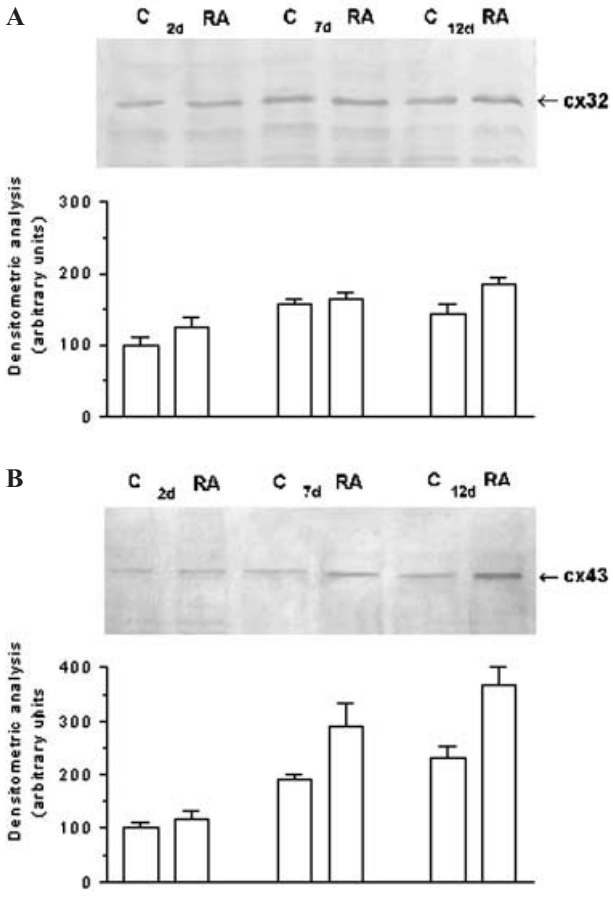

Figure 1. Western blot analysis of Cx32 (*A*) and Cx43 (*B*) in Hep G2 cells cultured for 2, 7 and 12 days in the absence (C) or presence of retinoic acid (RA). Densitometric evaluation of the bands, expressed as percentage of control at 2 days of culture, shows a strong increase in the amount of Cx43 (about 50 and 60%, respectively, after 7 and 12 days of RA treatment). Results are the average of three different experiments. The SD was less than 15%.

phorylated form in Hep G2 cells after 12 days of RA treatment (fig. 2A), while the increase in Cx43 phosphorylation was much less pronounced (fig. 2B).

In contrast, we observed no changes in Cx32 expression in fetal hepatocytes (fig. 3A) and no increase in its phosphorylated form could be detected after 48 h of RA treatment (fig. 3B).

To examine whether the increases in Cx concentration and phosphorylation induced by RA treatment in our systems were effectively correlated with improved insertion into cell membranes, as outlined above, we also examined the subcellular localisation of Cxs by indirect immunofluorescence. As figure 4 shows, in treated Hep G2 cells (fig. 4b, d), the Cxs considered showed the typical distribution in dotted and beaded lines at areas of cell-cell contact, indicative of well-assembled gap junctions. The effect was most evident after 12 days of RA treatment. In contrast, control cells displayed a smaller number of positive spots and diffuse cytoplasmic staining (fig. 4a, c).

In general, RA treatment markedly improved the localisation on cell membranes of the fluorescent spots when compared with controls, especially for Cx32 (fig. 4a, b). Quantitative evaluation also showed a strong increase in the number of Cx32-positive spots in treated cells (8.8 ± 2.2 spots/cell versus 5.1 ± 0.6 in controls). Moreover, in untreated cells, the diffuse staining observed in the cytoplasm, outside the cell-cell contact areas, suggests that the molecules in question were not properly inserted in the cell membrane. The positive effect of RA is also evident, albeit much less marked (20.4 ± 2.0 of treated cells

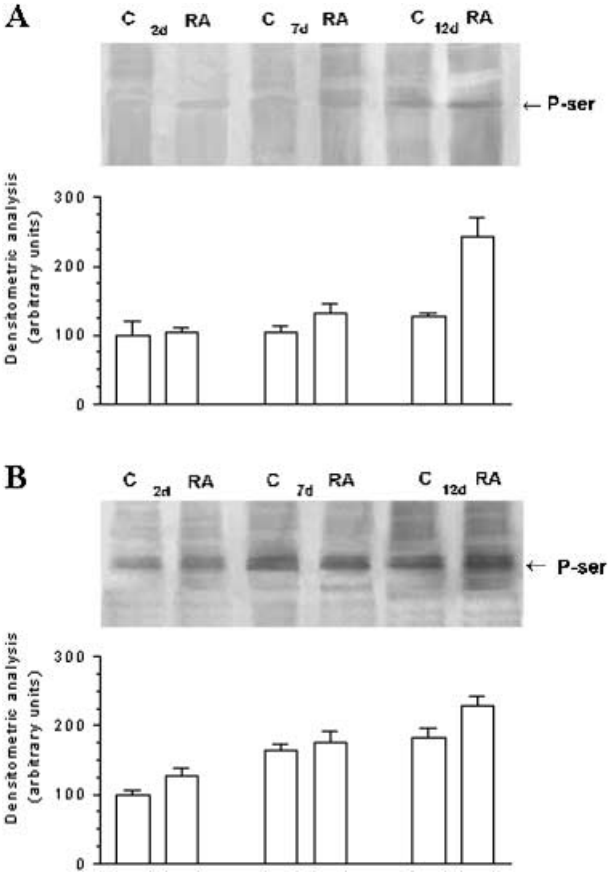

Figure 2. Western blot analysis of serine-phosphorylated Cx32 (*A*) and Cx43 (*B*) in Hep G2 cells cultured for 2, 7 and 12 days in the absence (C) or presence of retinoic acid (RA). Densitometric evaluation of the bands, expressed as percentage of control at 2 days of culture, clearly shows a strong increase in the amount of Cx32 phosphorylated after 12 days of RA treatment (about 90%). Results are the average of three different experiments. The SD is less than 15%.

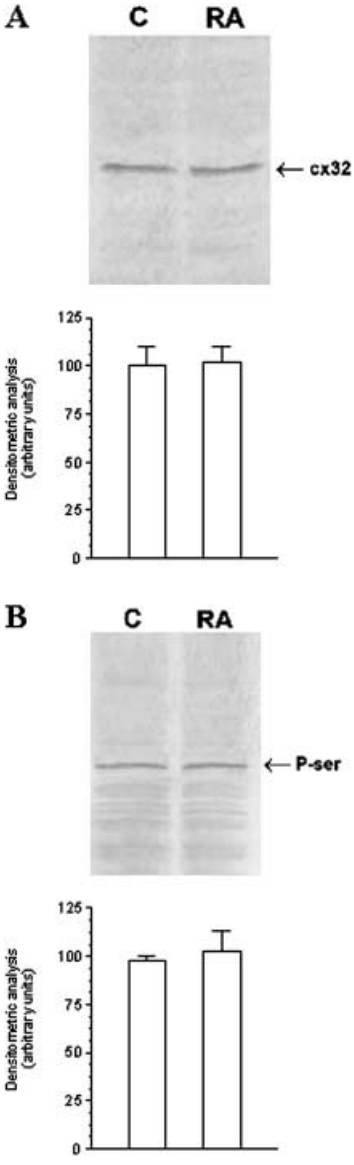

Figure 3. Western blot analysis of Cx32 (*A*) and serine-phosphorylated Cx32 (*B*) in fetal hepatocytes cultured for 48 h in the absence (C) or presence of retinoic acid (RA). Densitometric evaluation of the bands, expressed as percentage of control, shows no differences after RA treatment in either sample. Results are the average of three different experiments. The SD is less than 15%.

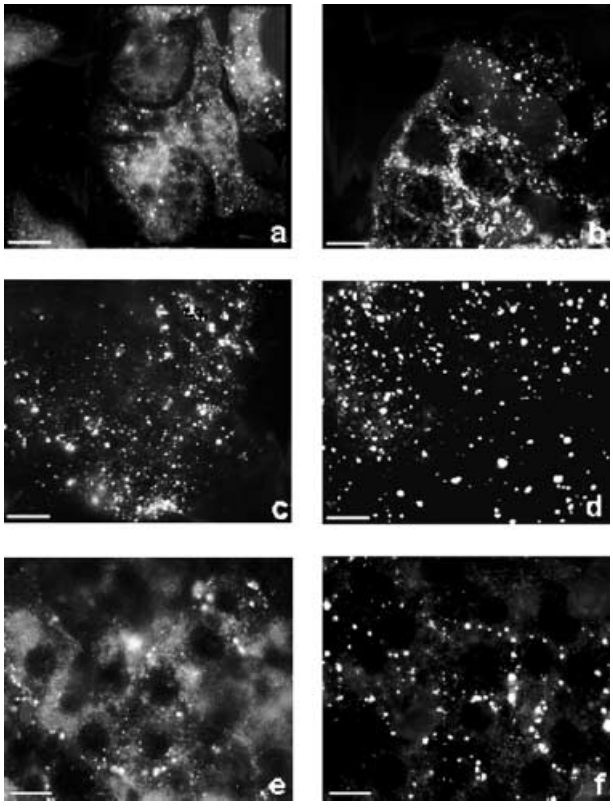

Figure 4. Immunostaining of Cx32 (*a, b*) and Cx43 (*c, d*) in Hep G2 cells after 12 days of culture in the absence (*a, c*) or presence (*b, d*) of RA, and in fetal hepatocytes after 48 h of culture in the absence (*e*) or presence (*f*) of RA. Improved localisation of fluorescent spots can be seen, presumably corresponding to gap junctions, on the plasma membrane of treated cells, compared with those of controls, where the fluorescence appears scattered in the cytoplasm. This pattern is particularly evident for Cx32 in Hep G2 cells. (bar, 10 µm).

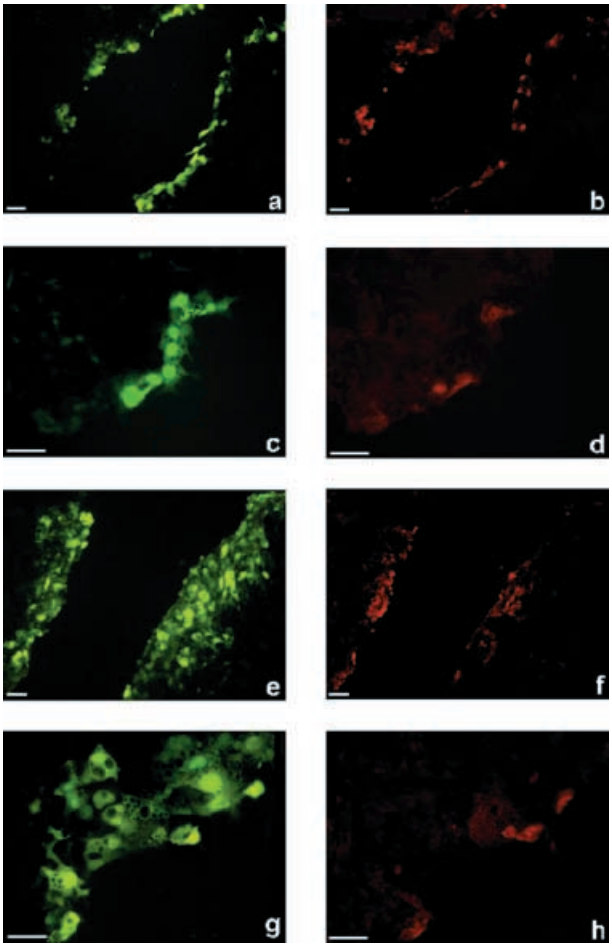

Figure 5. GJIC detected by the scrape-loading and dye transfer method in confluent monolayers of Hep G2 cells after 12 days of culture in the absence (*a–d*) or presence (*e–h*) of RA. Cells were loaded with Lucifer yellow (*a, c, e, g*) or rhodamine dextran (*b, d, f, h*). After RA treatment, a deeper layer of cells was stained by Lucifer yellow transferred through gap junctions than in controls. In controls, the cells stained by Lucifer yellow and rhodamine dextran correspond to those lying near the scrape and have been damaged (bar, 50 µm).

versus 14.2 ± 1.2 of controls), when the localisation of Cx43 is analysed.

In fetal hepatocytes, both immunofluorescence and quantitative analysis of Cx32-positive areas produced similar findings to those observed in Hep G2 cells (fig. 4e, f), although the differences between treated and control cells were less pronounced (6.9 ± 0.9 versus 4.4 ± 0.5).

Because the correct localisation and assembly of Cxs is not necessarily indicative of properly functioning gap junctions, we finally used the scrape-loading and dye transfer assay to verify the presence of functional GJIC [19]. Lucifer yellow and rhodamine dextran were introduced into cells in culture at non-cytotoxic concentrations. The first tracer dye does not diffuse through intact plasma membranes but its low molecular weight permits its transmission in a monolayer from one cell to another across gap junctions. In contrast, rhodamine dextran, a high-molecular-weight dye conjugate, can neither diffuse through intact plasma membranes nor cross the junctional channels, except through dead cells. The concurrent introduction of both Lucifer yellow and rhodamine dextran into cultures allows the identification of the communication-competent cells within a few minutes after loading.

As shown in figure 5, after RA treatment, Hep G2 cells showed evident Lucifer yellow dye-coupling in several layers adjacent to the scraped line, whereas rhodamine dextran was present only in the one-cell-thick layer consisting exclusively of damaged cells. In control cells, in contrast, both Lucifer yellow and rhodamine dextran dyes were restricted to the damaged cells along the two sides of the scraped line. Similar results were obtained with fetal hepatocytes (fig. 6).

Taken together, these data strongly suggest that RA exerts a positive effect on the regulation of GJIC in both experimental models studied; however, the mechanisms in-

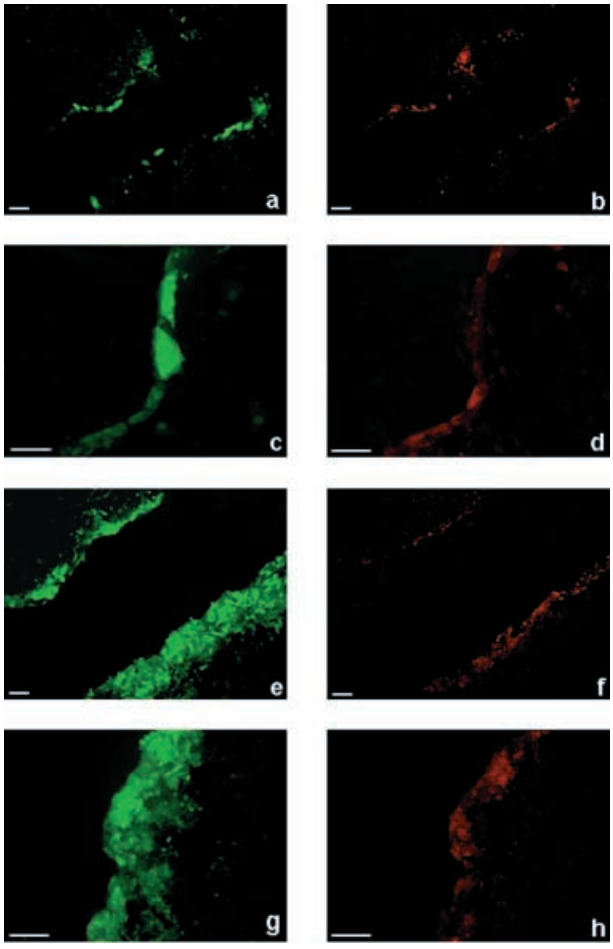

Figure 6. GJIC detected by the scrape-loading and dye transfer method in confluent monolayers of fetal hepatocytes after 48 h of culture in the absence (*a–d*) or presence (*e–h*) of RA. Cells were loaded with Lucifer yellow (*a, c, e, g*) or rhodamine dextran (*b, d, f, h*). After RA treatment, a deeper layer of cells was stained by Lucifer yellow transferred through gap junctions than in controls. In controls, the cells stained by Lucifer yellow and rhodamine dextran correspond to those lying near the scrape and have been damaged (bar, 100 µm).

volved are not identical. In Hep G2 cells, RA influences the expression, phosphorylation and localisation of Cx as well as gap junction functionality. In fetal hepatocytes, the effect of RA, although present, exclusively concerns the improved localisation of Cx32 in areas of cell-cell contact, and improved GJIC. No differences were observed in the expression levels or in the phosphorylation state of this protein.

Discussion

In this study we performed experiments to investigate the possible effects of RA on GJIC in two different models: the human hepatoma cell line Hep G2 and primary cultures of fetal hepatocytes. We demonstrated that treatment with RA can modulate GJIC positively, by acting directly on connexin expression and/or relocalisation. In addition, this effect is greater and somewhat more direct in Hep G2 cells than in fetal hepatocytes.

Alterations in channel-mediated cell-cell communication in transformed cells are widely described in the literature, in both in vitro and in vivo models. Such alterations may concern either the promotion or the progression stages of carcinogenesis; however, much attention has been directed towards the pre-neoplastic modifications of gap junction expression and function because of their reversibility. This step in neoplastic transformation is considered a good target for cancer prevention and thus represents a useful model to gain further insight into the mechanisms involved in the modification of GJIC.

In our experimental transformed model, Hep G2 cells, RA treatment induces a clear enhancement in Cx43 expression along with a much less consistent increase in Cx32. This result, which is not surprising because only Cx43 is typically expressed in Hep G2 cells, agrees with that found in F9 embryonal carcinoma cells [20] and in transformed mouse fibroblasts [21], in which Cx43 expression is up-regulated by retinoid treatment.

RA also enhances Cx43 expression in the rat liver epithelial cell line IAR203 [22]. In this case, RA is effective at very low concentrations and after a short time of treatment, suggesting a marked sensitivity of these cells to retinoids. In partial agreement with our results, in this model, the increase in the amount of protein is not paralleled by an increase in protein phoshorylation; the authors suggest that the up-regulation of Cx43 could be correlated with improved intercellular communication and that RA acts at the post-transcriptional level, by increasing protein stability.

The increase in Cx43 expression in Hep G2 cells could be interpreted as a reduction in their tumorigenicity; suppression of tumorigenicity has also been obtained in human carcinoma cells after transfection with this connexin [5].

Similarly, the increase in Cx32 expression, observed most markedely after 12 days of treatment, although slight, may also sustain the re-expression of a differentiated phenotype. In fact, Cx32 protein, which is normally present in non-transformed hepatocytes, is markedly reduced in hepatocellular carcinoma [2]. Moreover, transfection of human hepatoma cells with Cx32 induces a reduction in growth and tumorigenicity [3]. Also of considerable interest is the finding that the expression of Cx32 decreases significantly in chronic viral hepatitis and liver cirrhosis, and tends to decrease with the progression of liver injury [23]. Useful and more general therapeutic indications for treatment with RA could thus be suggested.

RA is thus able to affect the expression of both Cxs by acting at the post-transcriptional level. Although the ex-

act mechanisms involved must necessarily be assessed by further studies, one can hypothesise that RA regulates protein or mRNA stabilisation. A direct effect of RA at the transcriptional level can probably be excluded, because the specific responsive sites have not been found on the Cx genes [24].

The fact that intracellular levels of Cxs increase following RA treatment does not necessarily imply that these proteins are also properly assembled to form functional connexons. The organisation of Cx molecules into functional channels is very complex indeed. For example, an increase in Cx phosphorylation on serine has been correlated with Cx accumulation and positioning in plasma membrane plaques.

We therefore evaluated the amount of serine-phosphorylated Cx; the results showed that in Hep G2 cells, RA induces Cx43 and, more markedly, Cx32 phosphorylation. We suggest that RA may regulate the kinase/phosphatase ratio, which is responsible for the overall state of intracellular phosphorylation. However, we cannot exclude an increase in cAMP, which could influence the Hep G2 phosphorylation state as reported for the liver in vivo [25]. Our data suggest a further post-translational step in Cx modulation by RA, particularly evident for Cx32, as confirmed by immunostaining images that clearly show a more appropriate localisation of this protein on the surface of treated cells.

RA may also influence connexin relocalisation and positioning in the cell membrane of fetal hepatocytes. Fluorescent images of Cx32 have shown that in treated cells the immunostaining is localised particularly on the plasma membranes between juxtaposed cells. Moreover, the scrape-loading and dye transfer technique showed improved functionality of gap junctions. These effects are not directly related to the modulation of Cx32 expression or its phosphorylation state. To gain further insight into RA action in fetal hepatocytes, attention will need to be focused on different connexins (e.g. Cx26) or on the different mechanisms affecting gap junction sizing, assembly and gating. Nor can the effects mediated by growth factors or by intercellular adhesion molecules be excluded [26].

We used the scrape-loading and dye transfer technique for a more functional analysis of gap junctional communication. In Hep G2 cells, the extent of Lucifer yellow transfer and, thus, the ability of cells to communicate, was notably increased after RA treatment, a factor that could depend on both the Cxs under consideration. This enhanced capability is in agreement with a positive effect on metabolic exchanges and cell cooperation. Metabolic cell cooperation is a characteristic of well-differentiated cells and is known to be essential in vivo for a synchronised response of cells to extracellular stimuli, and, therefore, for tissue homeostasis. Gap-junctional communication is enhanced by RA in other cell types, including F9 embryonal carcinoma cells, where it seems to affect the stability of Cx43 mRNA [27]. RA is also likely to play a role in the gating process, by modulating the expression of proteins involved in the switching of channels between the open and closed states.

In summary, in Hep G2 cells, RA seems to regulate cell-cell communication by gap channels by acting at different levels: it regulates the expression and the phosphorylation state of Cxs; it modulates their insertion into plasma membrane plaques; it increases the level of GJIC, and by doing so, it helps to restore cell metabolic cooperation, preventing single cells from eluding signals involved in growth control. This, in turn, reflects the expression of a more differentiated phenotype, in line with our previous findings [13–15]. The diffusion of second messengers through gap channels is considered to be a major determinant for the establishment of metabolic coupling between neighbouring cells and for proper distribution of signals involved in the promotion of liver-specific functions.

A similar conclusion may also be extended to cultured fetal hepatocytes, although the effects of RA are certainly less explicit. Our data clearly indicate that treatment of fetal hepatocytes with RA induces better localisation and clustering of Cx32 into areas of cell-cell contact, improving gap junction functionality. Nevertheless, in this model, our experiments failed to demonstrate a direct effect of RA on Cx protein levels or differences in the state of phosphorylation. Although not completely predictable, these data agree with those published in the literature showing a lack of regulation of the phosphorylation level of Cx32 during liver development [28].

In conclusion, this study shows that the effects of RA on both normal and transformed hepatocytes may be related to its action on the cellular communication systems and provides clues for new strategies in the treatment of liver tumours.

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