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Analysis of *connexin43* gene expression induced by retinoic acid in F9 teratocarcinoma cells

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Abstract ***All-trans*** **retinoic acid (10^{-7} M) induces cell–cell communication and expression of the gap junction protein connexin43 in mouse F9 teratocarcinoma cells. Northern blot analysis revealed an increase of connexin43 mRNA after treatment with retinoic acid, accompanied by an increase of the mRNA of collagen IV, a differentiation marker. To address the question at what level gene expression is enhanced by retinoic acid, nuclear run-on experiments were carried out. There was no detectable change in the level of newly transcribed connexin43 mRNA. Therefore, we postulate a post-transcriptional mechanism responsible for the regulation of connexin43 mRNA levels by retinoic acid.**

Key words: Intercellular communication; Gap junction; Connexin43; Retinoic acid; Nuclear run-on

1. Introduction

Gap junctions are cell-to-cell channels allowing direct diffusion of ions and metabolites between mammalian cells. Several biological functions have been proposed, such as involvement in embryonic development, cell differentiation and growth control. Each channel is formed by docking and opening of hemichannels in adjacent cells consisting of six protein subunits, connexins, which are coded for by a multigene family [for review see [1]]. Intercellular communication mediated by gap junctions can be regulated by various agents; tumor promoting factors, in most cases, diminish cell–cell communication, whereas agents causing cell differentiation induce cell–cell communication.

Retinoic acid and some of its natural or synthetic derivatives exert a variety of biological effects necessary for normal cellular growth and differentiation [2]. The actions of retinoic acid are mediated by the retinoic acid receptors (RAR) and retinoid X receptors (RXR). Homo- or heterodimers of these receptors activate target genes through retinoic acid-responsive elements (RARE) in a ligand-dependent pathway [3]. The embryonal carcinoma cell line F9 is a model used to study mechanisms of differentiation induced by retinoic acid [4,5]. Retinoic acid leads to differentiation and converts F9 stem cells to a phenotype similar to cells of the primitive endoderm [6,7]. This process is accompanied by changes in cell morphology and in the expression of several genes, for example *collagen IV* and *laminin* [4,8]. Gap junctional communication is present in undifferentiated and differentiated F9 cells. Connexin43 and connexin26 mRNAs are detectable in stem cells and in differentiated F9 cells, whereas mRNA of connexin32 is not detectable in stem cells [9]. In other cell systems, we and others have shown that treatment of the cells with retinoic acid in physiological concentrations leads to an increase of intercellular communication [10–14] associated with an increased amount of connexin43 mRNA [10–13] or connexin43 protein [14].

2. Materials and methods

2.1. Cell culture

F9 cells [15] were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 2 mM glutamine and 20 μg/ml gentamicin on gelatinised plastic tissue culture dishes (Greiner, Frickenhausen, Germany). Differentiation was induced by addition of 1×10^{-7} M *all-trans* retinoic acid (Sigma, Deisenhofen, Germany), dissolved in ethanol. Controls received the same volume of solvent.

2.2. Gap junctional communication assay

Communication was assayed by microinjection of Lucifer yellow CH (10% in 0.33 M LiCl, w/v) into cells of confluent cultures by means of a microinjector and micromanipulator (Eppendorf, Hamburg, Germany). The number of fluorescent neighbors of the injected cells was scored after 5 min and serves as an index of junctional communication [13,16].

2.3. RNA isolation and Northern blot analysis

Total RNA was isolated from F9 cells by the guanidine thiocyanate method [17]. Twenty-five micrograms of total RNA was separated on 1% formaldehyde/agarose gels and blotted on Hybond N+ membranes as recommended by the manufacturer (Amersham, Braunschweig, Germany) and hybridised to a labelled 0.7 kb *Acc*I fragment of mouse connexin43 [18], kindly provided by Dr. Willecke (Bonn, Germany). β-Actin, used for reference, was measured by hybridisation with a radioactively labelled 0.6 kb *Eco*RI/*Hind*III fragment of the plasmid pMAβ-cod [19], kindly provided by Dr. Sakiyama (Japan). Collagen IV mRNA was assayed by hybridisation with a labelled 0.8 kb *Hind*III/*Pst*I fragment of the plasmid pTIVα [20], kindly provided by Dr. Oberbäumer (Berlin, Germany). Autoradiograph signals were quantitated by laser densitometry using GSXL software (Pharmacia, Freiburg, Germany).

2.4. Nuclear run-on analysis

Nuclei from F9 cells were isolated, and run-on transcription assays were performed by hybridising labelled nuclear RNA after chain elongation in the presence of [α-^{32}P]UTP to an excess (10 μg) of plasmids containing different cDNAs [18–20] immobilised on nylon filters [21–23]. The filters were subjected to autoradiography (1–3 days) after high-stringency washes and RNaseA treatment. Signals were quantitated by laser densitometry using GSXL software (Pharmacia, Freiburg, Germany).

2.5. Western blot analysis

10 μg of cellular proteins were separated by SDS-PAGE and electrotransferred onto a nylon membrane. After incubation with polyclonal rabbit antibodies against human connexin43, kindly provided by Dr. Rivedal (Oslo, Norway), detection was with the AURORA Western blot kit (ICN, Costa Mesa, USA).

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3. Results

Upon incubation of the embryonal carcinoma cell line F9 with *all-trans* retinoic acid (10^{-7} M), intercellular communication increased markedly above the solvent control, parallel to an increase in the amount of connexin43 protein (Table 1). In order to test if retinoic acid influences intercellular communication at the level of mRNA, the effect of retinoic acid on the amount of connexin43 mRNA was examined in Northern blots. After a 7 day treatment with retinoic acid at a concentration of 10^{-7} M, a 4.2-fold increase in connexin43 mRNA was observed (Fig. 1). To monitor the differentiation process caused by retinoic acid we hybridised the same RNA against collagen IV cDNA. Fig. 1 shows the typical increase in the level of the collagen IV mRNA during differentiation of F9 cells.

In nuclear run-on experiments using purified nuclei incubated in the presence of [^{32}P]UTP to monitor elongation of pre-existing RNA chains, labelled nuclear transcripts were isolated and hybridised to an excess of defined immobilised cDNAs. Fig. 2 shows the hybridisation of in vitro labelled RNA isolated from nuclei obtained from F9 cells treated with *all-trans* retinoic acid for 7 days or solvent as a control. β-*Actin* was included as a control, because the expression of this gene is not influenced by retinoic acid [24]. In comparison with β-*actin*, we observed a 3.1 ± 0.8-fold increase ($n = 3$) in the transcription rate of *collagen IV* after treatment with retinoic acid, whereas no significant alteration (1.1-fold) in the case of *connexin43* was detectable.

4. Discussion

Retinoic acid, at a concentration of 10^{-7} M, leads to differentiation of F9 stem cells into cells of the primitive endoderm. During this process we measured an increased intercellular communication, parallel to an increase in the amount of protein and mRNA of connexin43 (Table 1 and Fig. 1). In agreement with previous reports (e.g., [25,26]) we show that this differentiation process is accompanied by an up-regulated expression of the *collagen IV* gene at the transcriptional level (Figs. 1 and 2). As no alteration of the transcription rate of the *connexin43* gene was detected (Fig. 2), we postulate a post-transcriptional mechanism responsible for the activation of *connexin43* gene expression.

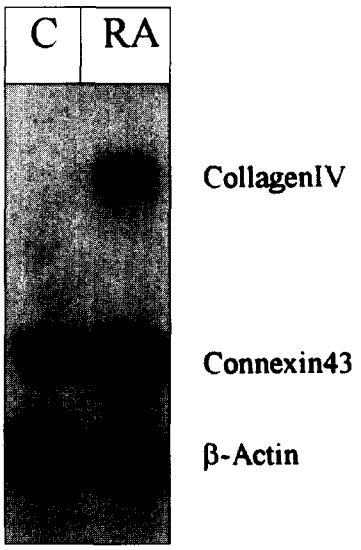

Fig. 1. Increase in connexin43 mRNA caused by retinoic acid in F9 embryonal carcinoma cells. Equal amounts of total RNA, isolated after a 7 day incubation of F9 cells with *all-trans* retinoic acid (RA, 10^{-7} M) or solvent as a control (C), were analysed by Northern blotting as indicated in Section 2. Exposure times were 1 h in the case of collagen IV and β-actin and 24 h in the case of connexin43.

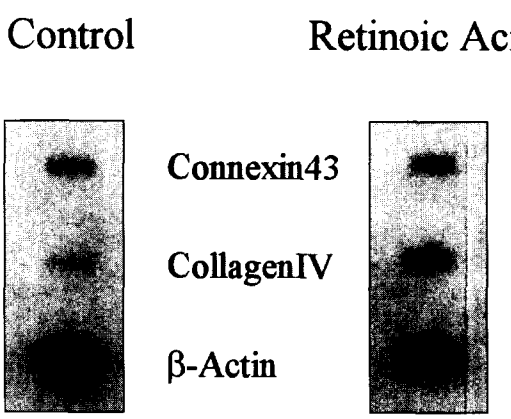

Fig. 2. Transcriptional activity of the *connexin43*, *collagen IV* and β-*actin* genes examined by nuclear run-on analysis in F9 cells after a 7 days treatment with *all-trans* retinoic acid. Plasmids containing cDNA inserts of the genes as indicated were used to hybridise to labelled nascent nuclear RNA as described under Section 2.

This result is reinforced by recent work on the promoters of the rat [27], human [28] and mouse [29] *connexin43* genes, where no putative RARE was identified. Since we did not observe an alteration of the transcription rate of the *connexin43* gene, we postulate that in F9 teratocarcinoma cells retinoic acid modulates the transcription of genes encoding proteins involved in post-transcriptional regulation, e.g., of connexin43 mRNA.

Our results differ in part from those by Bex et al. [14] obtained with the rat liver epithelial cell line IAR 203 cells. These authors treated IAR 203 cells with retinoic acid in concentrations of 10^{-7} M for 48 h and observed an increase of intercellular communication, an increase in the amount of the gap junction protein connexin43, but no increase in the level of connexin43 mRNA. This observation indicates that retinoic acid is also able to influence intercellular communication on the translational level, possibly by inducing cell adhesion molecules [30].

Table 1
Effects of *all-trans* retinoic acid (10^{-7} M) treatment on F9 teratocarcinoma cells on day 7

	n	Fold increase at day 7
Gap junctional communication[a]	3	2.0 ± 0.5
Connexin43 mRNA[b]	3	4.2 ± 1.7
Connexin43 transcription rate[c]	3	1.1 ± 0.3
Connexin43 protein[d]	2	3.0

Data are given as means ± SD.
[a]The number of communicating cells was 13.7 ± 3.4 vs. 6.9 ± 1.6 in the controls.
[b]See Fig. 1.
[c]See Fig. 2.
[d]Data from Western blot analysis.

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