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Gap junctional intercellular communication of cultured rat liver parenchymal cells is stabilized by epithelial cells and their isolated plasma membranes

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Abstract. The gap junctional intercellular communication (GJIC) determined by measuring dye coupling with Lucifer yellow, decreased within 3 d from 66% to 28% in monocultures of rat liver parenchymal cells. Coculturing of the parenchymal cells with a nonparenchymal epithelial cell line from rat liver resulted in increased and stabilized intercellular communication (83% after 3 d). The presence of isolated plasma membrane vesicles of the nonparenchymal epithelial cells also stabilized the intercellular communication between the liver parenchymal cells (70% after 3 d). When liver parenchymal cells were cocultured with a rat liver fibroblast cell line the gap junctional communication between the parenchymal cells was not stabilized (43% after 3 d), and isolated plasma membrane vesicles of the fibroblast were also unable to support the GJIC in parenchymal cells (35% after 3 d). It is concluded that plasma membrane constituents of the nonparenchymal epithelial cells were responsible for the stabilization of the GJIC between parenchymal cells. A heterotypic gap junctional communication between parenchymal and nonparenchymal cells was not observed.
Key words. Rat hepatocyte; gap junctional intercellular communication; coculture; plasma membrane; stabilization.

Isolated rat liver parenchymal cells (PC) in culture lose both their gap junctional intercellular communication (GJIC) and the gap junction-building proteins, the connexins[1]. Additional differentiated functions, like albumin secretion or the activity of enzymes implicated in the metabolism of xenobiotics, decrease with culture time and are lost within 2 weeks. On the other hand, these functions can be stabilized over 3 weeks by coculturing the PC with rat liver nonparenchymal epithelial cells (NEC)[2–4]. The exact origin of these cell lines has not yet been established, but it has been suggested that they originate from bile ductules[5]. Not all cell lines are able to stabilize PC in cocultures to the same extent[4]. Furthermore, it has been shown that a series of differentiated functions of PC are strongly dependent upon the actual cell density[6–9], although these effects are observed only under short-term conditions. As secreted factors have been shown not to be involved in this density-dependent regulation of differentiation, these studies implicate the importance of cell contacts. This has also been pointed out by Mesnil et al.[10], who showed that cell-cell contacts between PC and NEC are the major mechanism regulating the differentiated state of the PC.

Here we show by a direct approach that isolated plasma membranes as well as fixed feeder layers of NEC were able to stabilize the GJIC of PC in culture, whereas when a non-supporting cell line, rat liver fibroblasts, was used for coculture with PC, the GJIC between the PC decreased. Furthermore, isolated plasma membrane vesicles from the fibroblasts were also unable to stabilize the communication between the PC.

Materials and methods

Isolation of rat liver parenchymal cells (PC). PC were isolated from male Sprague-Dawley rats (220–280 g) according to the method of Wang et al.[11] with the modifications described by Utesch and Oesch[4].

Rat liver nonparenchymal epithelial cells (NEC, clone 1) and rat liver fibroblasts (RLF) were isolated and cultured as described[4].

Monoculture of PC. Freshly isolated PC were seeded in DMEM with 10% FCS, 1 mM dexamethasone at 6–7 × 10^4 cells/cm^2 onto culture dishes previously coated with collagen IV, and cultured at 37 °C in a humidified atmosphere with 5% CO_2. The medium was aspirated off 2 h after plating the cells to remove unattached cells and then changed daily.

Coculture of PC with NEC or RLF. PC were seeded as described at 4–5 × 10^4 cells/cm^2. After 2 hours 6–7 × 10^4 NEC or 4 × 10^4 RLF per cm^2 in DMEM were added.

Culture of PC on fixed monolayers of NEC. One day after reaching confluency the NEC monolayers were washed twice with PBS and fixed with ice-cold ethanol/acetic acid (3/1, v/v) for 1 hour. The fixed monolayer was washed 5 times with PBS and 5 × 10^4 PC/cm^2 were added in DMEM. Unattached PC were removed after 6 h by medium change.

Dye injection and dye transfer assay. Microinjection was performed using a micromanipulator 5170, a pneumatic microinjector 5242 and microcapillaries (Femtotips) from Eppendorf (Hamburg, Germany). The capillaries were backloaded with 5% Lucifer yellow CH in 0.1 M lithium chloride. The injection was followed under a

Fluovert microscope (Leitz, Wetzlar, Germany, E3 filter combination, magnification 384×, or Zeiss, Oberkochen, Germany, filter combination no. 487906, magnification 320×). For evaluation of the GJIC the injected cell was observed for at least 2 min after injection for dye transfer to surrounding cells. A minimum of 10 PC per culture dish were injected. One culture dish was examined for maximal 30 min under conditions of room temperature and normal atmosphere. Within this period no influence on the dye coupling was observed. Dye coupling was calculated by dividing cases of dye coupling by the number of injections and multiplying by 100 to give the percentage of microinjections which lead to dye coupling.

Preparation of plasma membranes from NEC and RLF. Plasma membranes were isolated from confluent cultures of NEC or RLF as described[12]. The isolated plasma membranes were concentrated either by ultrafiltration over an Amicon filter XM300 or by ultracentrifugation at 100,000 × *g* for 1 hour at 4 °C, which was repeated a second time after resuspension of the pellet in 0.9% NaCl. The isolated and concentrated plasma membranes were stored at −80 °C until use.

Miscellaneous methods. The protein content was measured using the modified Lowry method[13] with serum albumin as a standard.

Results and discussion

GJIC decreased continuously in confluent monocultures from 66% of dye coupling on day 1 to 28% on day 3 (table). After 4–6 d cell borders disappeared and the monolayer disintegrated. These observations are in good agreement with results obtained by others showing that, although PC are extensively coupled by gap junctions in vivo, monocultures of PC lose differentiated functions within a short time[10,14,15]. On the other hand, coculturing PC with NEC not only resulted in stabilization of the GJIC, but also led to an increase of junctionally coupled cells (table).

Several molecular mechanisms could be responsible for this effect.

1) Heterotypic junctional communication between PC and NEC. In addition to NEC, a series of other cells have been tested for heterotypic junctional communication in coculture with PC: rat liver Kupffer, endothelial and oval cells, RLF and mouse fibroblasts (C3H/10T1/2), FAO Reuber hepatoma cells, 9FRS and hybrid cells. Gap junctional communication of these cells with PC was observed only in the case of a hybrid cell line (hybrids between PC and hepatoma cells, obtained from Dr. Petzinger, Giessen, Germany). This finding is in agreement with observations by Mesnil et al.[10].

2) Soluble compounds secreted by the NEC could also have led to stabilisation of GJIC. However, addition of conditioned medium from NEC to PC was without any effect on GJIC of PC (23% dye coupling after 3 d). The same was true for distance cultures of PC and NEC, where cell-cell contacts were avoided, but soluble factors could diffuse between the cell compartments.

3) Stabilization by cell-cell contacts between NEC and PC via specific cell surface molecules. Nakamura et al.[16] showed that in mature rat hepatocytes both high cell density and the addition of isolated liver plasma membranes to sparsely seeded hepatocytes were able to stabilize differentiated functions such as induction of tyrosine aminotransferase, serine dehydratase and malic enzyme, and synthesis of triglycerides. Recently, Corlu et al.[17] showed in cocultures of PC and NEC that monoclonal antibodies against plasma membrane proteins interfered with the stabilizing effect, suggesting that the stabilization of differentiation observed in cocultures is a cell contact-mediated event.

Starting with the results mentioned above, we asked the question whether GJIC is a differentiated function of PC which is also stabilized by cell contact-mediated mechanisms. In a first approach, PC were cultured on ethanol/acetic acid fixed monolayers of NEC. As shown in the table, a stabilizing effect of GJIC between the PC can be observed. Concomitantly with the loss of this stabilization a disintegration of the NEC feeder-layer occurred. The disintegration was independent of the fixatives used (formaldehyde, carbodiimide, p-bezoquinone or β-irradiation) and of the absence or presence of the protease inhibitor soyabean trypsin inhibitor. It is suggested that as the feeder-layer disappears, a supportive effect on the GJIC is lost.

Dependency of GJIC between PC on the cell culture design.

Cell culture design	dye coupling (%) day 1	day 3
PC monoculture	66 ± 14	28 ± 17
PC + NEC coculture	96 ± 5**	83 ± 19**
PC cultured on fixed NEC monolayer	85 ± 15	45 ± 8
PC + NEC plasma membranes (100 µg/ml)	97 ± 6**	70 ± 19**
PC + RLF cocultures	70 ± 26	43 ± 30
PC + RLF plasma membranes (100 µg/ml)	58 ± 10	35 ± 6

Values are the means ± SD of at least 3 independent experiments with 1 dish per experiment and at least 10 microinjected cells/dish/experiment. Statistical evaluation was done with Dunnett's Test comparing PC monoculture on day 1 or 3 with all other cell culture designs, respectively. Significantly different at *: $p \leq 0.05$; **: $p \leq 0.01$.

The supportive effect of the feeder-layer pointed to the involvement of plasma membrane-associated compounds. Therefore, isolated plasma membranes of NEC were added to PC, and their influence of GJIC between the cultured PC was measured. In a range-finding experiment it was shown that increasing amounts of plasma membranes of NEC increased the stabilization of the GJIC of PC. After 3 days in culture, the degree of GJIC was similar to that observed in coculture with NEC (table) and much higher than that in monocultures. This effect was observed only when the plasma membranes were added daily at each medium renewal. As a specific control a non-supporting cell line (RLF) was used for coculture with PC, and also isolated plasma membrane preparations of the RLF were added to PC cultures. Neither the RLF cells nor their plasma membranes were able to support the GJIC between the PC (table).

These results support the observations that cell-cell contacts are responsible for the stabilization of differentiated functions of PC. The stabilization of the PC is dependent upon the cell line which is used for coculture. Further experiments should identify the molecules involved in this process.

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