Alterations in Intercellular Communication during the Stage of Promotion (43308)

JAMES E. KLAUNIG[1]

Department of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, Indiana 46202

Rodent hepatic tumorigenesis follows a stepwise procession of stages from an early modified preneoplastic hepatocyte to the formation of a malignant neoplasm (1). The promotion stage involves the selective clonal expansion of the initiated cell through cell division. In the liver, this clonal expansion is demonstrated morphologically by phenotypic differences from normal liver (the hepatic focus). The process of promotion seems to involve modification of gene expression, not genetic damage, and appears to be reversible. Although a number of endogenous and exogenous agents and factors have been identified as hepatic tumor promoters, the mechanisms by which the selective clonal expansion of the initiated hepatocyte occurs are still not completely resolved. Agents that have activity in the promotion stage of tumor formation have several characteristics in common, including the ability to induce DNA synthesis and cell proliferation in the target cells. Another common effect of tumor-promoting agents appears to be their ability to disrupt and inhibit gap junctional intercellular communication (2). Gap junctions are plasma membrane-bound organelles that form intercellular conduits between adjacent cells of a tissue. The gap junction appears to be important in the normal growth control, differentiation, and homeostasis of the hepatocyte (3). During liver tumor promotion, hepatic gap junctions and hepatic gap junctional intercellular communication are modified. This modification may be important in the selective isolation of the preneoplastic, initiated hepatocytes from the normal, surrounding liver by effectively blocking the transmission of growth control signals from the normal hepatocytes (4, 5).

Stages of Hepatic Carcinogenesis

The induction of cancer in the liver by chemicals is a very complicated process that involves the induction of mutations in hepatocytes and the selective growth of these mutated cells to neoplasia. Three distinct stages of the chemical carcinogenesis process have been proposed: initiation, promotion, and progression (1). The initiation stage is the first event in the carcinogenesis process involving the mutation of the genetic material of the target hepatocyte. The "locking in" of the mutation(s) requires at least one round of replicative DNA synthesis. Promotion involves the selective clonal expansion of the initiated cell via cell proliferation. Promotion may occur through endogenous intrinsic mechanisms or via exogenous agents. Endogenous factors functioning at this stage of tumorigenesis include hormones and growth factors. Exogenous agents include a diverse variety of chemical and physical compounds that all share in their ability to elicit the selective proliferation of the initiated cells. Promotion appears to be reversible at least during the early period of this stage. The progression stage is the least understood of the three stages. It involves the modification of the initiated cell population from the preneoplastic stage to the neoplastic stage. This appears to include the acquisition of additional genetic-damaging events, as well as chromosome alterations in the preneoplastic cell population, that results in the formation of neoplastic lesions.

Rodent hepatic tumorigenesis involves the expression of several well-defined and morphologically distinct stages. This allows for the placement of the initiation, promotion, and progression stages of carcinogenesis into morphologic, demonstrable lesions. The earliest morphologically distinct lesion in the rodent liver is the focal area of cellular alteration, also called the altered hepatic focus or focus. The focus is a collection of cells phenotypically distinct from normal hepatocytes that appears to be clonally derived from a single cell. The focus is the clonally expanded initiated cell and, therefore, represents the promotion stage of hepatic carcinogenesis. The next morphologically distinct stage of tumor formation in the rodent liver is the neoplastic stage, comprising both benign neoplasms (adenoma) and malignant neoplasms (carcinoma). The neoplastic stage is not reversible. The succession from focus to neoplasm involves the acquisition of additional

[1] To whom requests for reprints should be addressed at Department of Pharmacology and Toxicology Medical Research Facility 003, Indiana University School of Medicine, 1001 Walnut Street, Indianapolis, IN 46223.

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genetic changes in the altered cell population and, thus, corresponds to the progression stage of carcinogenesis.

While no additional genetic damage in the initiated hepatocyte occurs during the promotion stage, modification of gene expression does occur. Both endogenous and exogenous factors have been identified that can control the clonal expansion of the initiated cell and alter hepatocyte gene expression. While diverse in chemical form, these factors have common biological effects on the initiated cell population. A major effect is the clonal expansion of the initiated cell. This can be achieved by increasing DNA synthesis and cell division, reducing the death rate (loss of cells in the focus by apoptosis), or a combination of both (6). While the promotion activity of endogenous and exogenous agents can be defined with regard to the eventual effect on the initiated cell population, that is, expansion of the initiated cell population, the cellular mechanisms by which this process is put forth are still unresolved. One cellular effect that has been demonstrated by a number of agents that express hepatic tumor-promoting activity is the blockage or inhibition of gap junctional intercellular communication (7).

Gap Junction Structure

Gap junctions are cellular organelles located in the plasma membrane. These structures allow for the transfer of low molecular weight materials (under 1,000 kDa) between the cytoplasms of adjacent cells (8). Each gap junctional plaque consists of numerous small pores, referred to as connexons, that provide the functional conduit for the transfer of materials. The connexons appear to be made up of six homologous proteins that line the pore. The gap junction proteins transverse the plasma membrane 4 times, starting and ending in the cytoplasmic face. Three distinct gap junction proteins have been defined to date and have been referred to as connexins. These proteins have different molecular weights and are expressed in different tissues. For the purpose of this discussion, they will be referred to as connexin 43, connexin 32, and connexin 26, based on their mol wt of 43,000, 32,000, and 26,000, respectively (9). In the liver, all three connexin proteins are expressed. Hepatocytes express predominantly connexin (CX) 32 and to a lesser extent connexin 26, whereas the nonparenchymal elements express predominantly connexin 43.

Gap Junctional Intercellular Communication

The transfer of materials between adjacent cells through the gap junctions is referred to as gap junctional intercellular communication (GJIC). GJIC involves passive transport and has been measured a number of ways, including by dye coupling (transfer of microinjected florescent dye from one cell to an adjacent cell), electrical coupling, and metabolic cooperation (7). GJIC is regulated through two major pathways. Gap junction protein is transcribed, translated, incorporated in the plasma membrane, and degraded. Any agent that effects the synthesis and turnover of the gap junction protein will influence GJIC. A second control pathway involves the local regulation of the opening and closing of the gap junction pore. This pathway appears to be under the control of a number of intracellular factors, including intracellular free calcium concentration, intracellular hydrogen ion concentration, cAMP concentration, phosphoinositol concentration, adhesion proteins, and microtubule integrity. Modification of these factors by chemical agents may influence GJIC (10–13).

Role of Gap Junctions in Tumorigenesis

A number of physiologic functions have been subscribed to hepatic gap junctions, including maintenance of homeostasis, maintenance of differentiation, and growth control (5, 7). Blockage of GJIC, therefore, would result in the loss of growth control, differentiation, and homeostasis. One working hypothesis on the role of gap junctions and GJIC in the cancer process is that the disruption and down-regulation of gap junction protein expression and/or GJIC in tumor cells allow the neoplastic cells to grow through the loss of normal cell to cell growth regulation. This is supported in part by the well-known characteristic of the loss of cell to cell contact inhibition in tumor cells (14). Therefore, one would expect that GJIC is absent or markedly decreased in tumor cells. However, from reports on GJIC expression in tumor cells, a disparity exists. While a number of reports have shown that cells in tumor tissues have decreased gap junctions and decreased GJIC (15–18), other studies have suggested that tumor cells have both normal-appearing gap junctions and normal GJIC (7). Differences in these studies may be related in part to the source of the tumor tissue. Some of the studies in which tumor cells show normal expression of gap junctions and normal GJIC employ tumor cell cultures as their model and may represent an *in vitro* artifact from using ill-defined tumor cell lines and tumor cell lines that no longer biologically resemble the primary tumor from which they were derived. Studies with primary hepatic tumors from rodents have shown that both adenomas and carcinomas have decreased gap junctions (as evidenced by decreased immunohistochemical staining), decreased mRNA for the gap junction protein, and decreased GJIC (15–18). In addition, cells isolated from the primary tumors show decreased GJIC and decreased mRNA for the gap junction protein connexin 32 (12). While the differences seen by different investigators on GJIC and gap junction protein expression in tumor cells may be in deference to the hypotheses noted above, more recent findings have suggested that a related, but alternative, hy-

pothesis on the role of gap junctions in cancer might be more appropriate. In this postulate, it is suggested that GJIC is important during the promotion stage of carcinogenesis in that the blockage of GJIC and the reduction of gap junction protein expression by endogenous or exogenous promoters isolate the initiated, preneoplastic cells from the normal, surrounding hepatocytes. This isolation of the preneoplastic cells results in the loss of the transfer of growth control signals from the normal cells and the accumulation of growth stimulatory signals in the initiated cell population (5). Whatever the mechanism, the result is an increase in DNA synthesis and cell division of the preneoplastic cell population, resulting in the clonal expansion of these cells. The postulate that the loss of gap junctional protein expression and GJIC is important in the promotion stage of carcinogenesis is supported by the findings that gap junction protein expression is decreased in rat (16) and mouse (18, 20) liver foci. Similarly, a decrease in GJIC (dye coupling) between cocultured normal rat hepatocytes and hepatocytes isolated from preneoplastic rat liver foci has been shown (19, 20). A minimal level of GJIC was seen between the preneoplastic hepatocytes and the normal hepatocytes. After treatment of the cultures with the hepatic tumor promoter phenobarbital, the level of GJIC was completely absent. In contrast, normal hepatocyte to normal hepatocyte and preneoplastic hepatocyte to preneoplastic hepatocyte GJIC were almost 100%. In cells isolated from hepatic adenomas and co-cultured with normal hepatocytes, no discernible GJIC was detectable between the two cell types, whereas dye coupling was evident between normal to normal cells (20). GJIC between adenoma hepatocytes was evident, but considerably reduced. In addition, neoplastic hepatic lesions microinjected with the fluorescent dye Lucifer yellow CH displayed minimal but measurable GJIC (measured by dye coupling) between the neoplastic cells, but the injected dye failed to spread from the neoplastic cells to the normal surrounding cells. These results support the hypothesis that selective GJIC is evident between hepatocytes in neoplastic-preneoplastic lesions and the normal surrounding hepatocytes.

Effect of Promoting Agents on Gap Junctions

A number of endogenous or exogenous hepatic tumor promoters have been examined for their effects on hepatic GJIC (Table I). The inhibition of hepatic GJIC by exogenous and endogenous agents appears to be limited, with a few exceptions, to chemicals with hepatic tumor-promoting properties. In cultured hepatocytes, these agents inhibit GJIC in a dose-dependent manner. The inhibition of GJIC does not appear to be simply a cytolethal response and is not seen with classical cytolethal agents at lethal concentrations (23).

In addition to *in vitro* studies, *in vivo* evidence has shown that both hepatic gap junction cx32 protein expression in the plasma membrane (12, 16, 18) and GJIC are decreased in neoplastic and preneoplastic liver lesions. In neoplastic lesions, the decrease in gap junction cx32 protein expression accompanies a decrease in expression of mRNA for cx32. In preneoplastic rat hepatic foci, the decrease in protein expression is dependent, at least in part, on the continued exposure of the animal to a tumor-promoting compound (16). With removal of the hepatic tumor promoter, many of the rat preneoplastic foci showed normal expression of cx32 (16).

We have made similar findings in the mouse liver (Tables II and III). Male B6C3F1 mice received a single dose of diethylnitrosamine at 30 days of age. After 6

Table I. Hepatic Tumor Promoters Shown to Inhibit Hepatic Gap Junctional Intercellular Communication

Compound	Reference
Pesticides	
Dieldrin	28
DDT	24
Lindane	〃
Endosulfan	22
Chlordane	〃
Heptachlor	〃
Paraquat	23
Pharmaceuticals	
Phenobarbital	21
Sodium barbital	32
Diazapam	25
Lovastatin	31
Peroxisome proliferators	
Diethylhexylpthalate	27
Trichloroethylene	26
Tetrachloroethylene	〃
Trichloroacetic acid	〃
Nafenopin	30
Clofibric acid	30
Oxygen Radical-Generating Compounds	
Xanthine/xanthine oxidase	29
Hydrogen peroxide	29
Glucose/glucose oxidase	29

Table II. Cx32 Expression in Mouse Hepatic Foci[a]

Foci	− Phenobarbital (%)	+ Phenobarbital[b] (%)	+ Phenobarbital − phenobarbital[c] (%)
Deficient in cx32	9	76	14
Normal expression of cx32	91	24	84

[a] Foci (100) were counted at random in 10 B6C3F1 mice per group.
[b] Phenobarbital was given for 28 days, then the mice were sampled.
[c] Phenobarbital was given for 28 days, then phenobarbital was removed for 14 days, and then the mice were sampled.

Table III. Cx32 Expression in Mouse Hepatic Adenomas[a]

Adenomas	− Phenobarbital (%)	+ Phenobarbital[b] (%)	+ Phenobarbital − phenobarbital[c] (%)
Deficient in cx32	100	98	100
Normal expression of cx32	0	2	0

[a] Fifty adenomas were counted at random in 10 B6C3F1 mice per group.
[b] Phenobarbital was given for 28 days, then the mice were sampled.
[c] Phenobarbital was given for 28 days, then phenobarbital was removed for 14 days, and then the mice were sampled.

months, mice received either no treatment, phenobarbital in their drinking water for 28 days, or phenobarbital in their drinking water for 28 days, followed by no treatment for 14 days. Mice were sampled after the treatment periods and examined for connexin 32 immunohistochemical staining in hepatic foci (Table II) and in hepatic adenomas (Table III). In mice not treated with phenobarbital, most of the foci showed normal (compared with the surrounding liver) expression of gap junction protein. Only approximately 10% of the focal lesions were deficient in connexin 32 staining (Table II). All adenomas from this same group of mice displayed decreased connexin 32 expression (Table III). In mice that received phenobarbital for 28 days, over 75% of the foci showed a decreased expression of connexin 32 staining (Table II). Over 98% of the adenomas examined from this group also showed a decreased staining for connexin 32 (Table III). In mice that received phenobarbital for 28 days and then were removed from phenobarbital exposure for 14 days prior to sampling, most of the foci (84%) showed normal expression for the connexin 32 protein (Table II). In contrast, all of the adenomas examined in these mice were deficient for connexin 32 staining (Table III).

Tumor promoters may effect GJIC through at least two different mechanisms. One means is through modification of normal cellular control pathways for the opening and closing of the gap junction channel. In this case, the tumor promoter may influence intracellular pH, calcium, cAMP, direct interaction with the gap junction protein, or other intracellular gap junction controls (Klaunig and Ruch, 1989; (7). This may be the mechanism seen in cultured hepatocytes and *in vivo* liver after acute exposure to a tumor-promoting compound. This inhibition of GJIC appears to be rapidly reversible and dose dependent. A second mechanism of tumor promoter inhibition of GJIC may occur through modification of transcription and/or translation expression levels of gap junction protein. This is frequently seen in neoplastic hepatic lesions. The reversibility of this effect is unknown *in vivo*, but this down-regulation of hepatic gap junction protein expression appears to be an attribute acquired with liver neoplasia or in preneoplastic hepatic foci in animals treated with a tumor promoter.

Summary

The promotion stage is a crucial step in the process of carcinogenesis. During this stage, the initiated cell population is clonally expanded to morphologically discriminable forms. Exogenous or endogenous agents that influence this clonal expansion have tumor-promoting activity. Inhibition of gap junctional intercellular communication is one of a number of cellular changes seen in cells after exposure to promoting agents. GJIC can be inhibited through either modification of intracellular control mechanism or through transcriptional or translational down-expression of the gap junction protein. Through either mechanism, the net effect is a decrease in GJIC by tumor promoters. This decrease in GJIC, while occurring in normal cells and preneoplastic cells alike, appears to be more efficacious in the preneoplastic cells, and appears to prevent GJIC between the preneoplastic cells and the normal surrounding hepatocytes. This isolation of the preneoplastic cells by hepatic tumor promoters from the normal surrounding hepatocytes may separate the preneoplastic cells from growth regulatory control of the normal liver, thus allowing the preneoplastic cells to clonally expand by cell proliferation. Whether the disruption of GJIC and down-regulation of gap junction protein expression seen in hepatic foci by exposure to tumor promoters are causes or effects of the resulting cell proliferation remains to be determined. Certainly, the modification of GJIC and the expression of the gap junction protein by tumor promoters are important cellular changes that produce a phenotypically altered population of hepatocytes.

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