Session 7.6
Non-genotoxic carcinogenicity: Mechanistic perspectives for alternatives

Cancerous Contradictions: The Mis-Regulation of Human Carcinogens Based on Animal Data*

Andrew Knight¹, Jarrod Bailey² and Jonathan Balcombe³
¹Animal Consultants International, London, UK; ²Faculty of Medical Sciences, University of Newcastle upon Tyne, UK; ³Physicians Committee for Responsible Medicine, Washington DC, USA

Summary
The regulation of human exposures to potential carcinogens constitutes society’s most important use of animal carcinogenicity data. However, for environmental contaminants of greatest U.S. concern, we found that in most cases (58.1%; 93/160) the U.S. Environmental Protection Agency (EPA) considered the animal data inadequate to support a classification of probable human carcinogen or non-carcinogen.
The World Health Organisation’s International Agency for Research on Cancer (IARC) is a leading international authority on carcinogenicity assessments. For chemicals lacking human exposure data (the great majority), IARC classifications of identical chemicals were significantly more conservative than EPA classifications (p<0.0001), indicating that: (i) the EPA is over-reliant on animal carcinogenicity data, (ii) as a result, it tends to over-predict carcinogenic risk, and (iii) the true predictivity for human carcinogenicity of animal data is even poorer than indicated by EPA figures alone. EPA policy erroneously assuming that tumours in animals are indicative of human carcinogenicity is implicated as the greatest source of these errors.

Keywords: animal experiment, animal test, bioassay, cancer prevention, carcinogenicity, chemical classification, chemical safety, risk assessment

Introduction
Since the first animal carcinogenicity test in 1915, when Yamagiwa and Ichikawa showed that coal tar applied to rabbits’ ears caused skin carcinomas, several thousand carcinogenicity bioassays have been conducted, with the objective of determining human carcinogenic risks for the great majority of chemicals lacking human exposure data (Huff, 1999). However, animal carcinogenicity testing remains a controversial area of research. Proponents claim that all known human carcinogens that have been studied in sufficient animal species have produced positive results in one or more species (Wilbourn et al., 1986; Tomatis et al., 1989; Rall, 2000). Critics respond that if enough animal testing is conducted, carcinogenesis will eventually occur in some species, regardless of human cancer risk. A study published in Mutagenesis found that of 20 human non-carcinogens, 19 produced carcinogenic effects in animals (Ennever et al., 1987).
The most important use of animal carcinogenicity data lies in the regulation of human exposures to potential carcinogens. The U.S. Federal agency most responsible for regulating exposures to environmental contaminants is the Environmental Protection Agency (EPA, undated a), and the chemicals of greatest public
health concern (EPA, undated b) are listed within its Integrated Risk Information System (IRIS) chemicals database, along with their animal toxicity data and consequent human carcinogenicity assessments (EPA, undated c).

To assess the utility of animal carcinogenicity data in deriving human carcinogenicity assessments, we surveyed the IRIS chemicals database. To assess the reliability of the EPA carcinogenicity assessments obtained from animal test data, we compared them with those of a leading world authority, the World Health Organization’s International Agency for Research on Cancer (IARC).

Methods

The 543 chemicals catalogued in the EPA’s IRIS chemicals database (as of January 1, 2004; EPA, undated d) were examined to determine the proportion for which the EPA was able to derive classifications of “probable human carcinogen” or “probable human non-carcinogen” based primarily on animal carcinogenicity data. The relatively few classifications of “definite human carcinogen” relied primarily on available human exposure data. The remaining classifications of “unclassifiable” or “possible human carcinogen” were not considered substantially useful for risk assessment or regulatory purposes. They are excluded from the U.S. National Toxicology Program annual Report on Carcinogens (NTP, 2002).

Of the 177 chemicals considered by the EPA to possess at least limited human or animal data, 128 were assigned human carcinogenicity classifications by both the EPA and the IARC. Of these 128, 17 were considered by the EPA to possess at least limited human data, while 111 were primarily reliant on animal data.

The consistency of classifications between the EPA and the IARC was examined for these two groups by comparing the carcinogenicity classification proportions within each group by chi-square tests, and also by comparing the individual classifications of the 111 chemicals primarily reliant on animal carcinogenicity data.

Chi-squared tests provide statistical calculations of the probability that two data sets, such as EPA and IARC human carcinogenicity classifications, are samples from the same underlying data population, and that any observed differences are simply due to random sampling variation. Large chi-squared (X²) values reflect increased probabilities that observed differences are due to real differences in underlying data populations.

Results

EPA human carcinogenicity classifications

Of the 543 chemicals catalogued in the EPA’s IRIS chemicals database, 235 had been assigned human carcinogenicity classifications. Of these, 17 were classified as definite (A) or probable (B1) human carcinogens on the basis of their human carcinogenicity data. Of the remaining 218 chemicals lacking even limited human data, 160 were deemed to possess animal carcinogenicity data, primarily sourced from the biomedical literature (B2, C, subset of D, and E; tab. 1).

The human utility of animal carcinogenicity data based on EPA figures

Of the 160 EPA chemicals lacking even limited human data (A or B1) but having animal data (B2, C, subset of D, and E), 64 were considered probable human carcinogens (B2), and three were considered probably not carcinogenic to humans (E). The remaining 93 chemicals were considered possible human carcinogens (C; 40) or unclassifiable as to their human carcinogenicity (D; 53) based on animal data considered inadequate to support a stronger classification (tab. 1).

In sum, of those 160 chemicals lacking even limited human data population, and that any observed differences are simply due to random sampling variation. Large chi-squared (X²) values reflect increased probabilities that observed differences are due to real differences in underlying data populations.

Tab. 1: EPA human carcinogenicity classifications of IRIS chemicals

<table>
<thead>
<tr>
<th>EPA human carcinogenicity classification (with basis for classification)</th>
<th>No. of chemicals</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: Human carcinogen (convincing human data)</td>
<td>11</td>
<td>4.7</td>
</tr>
<tr>
<td>B1: Probable human carcinogen (limited human data)</td>
<td>6</td>
<td>2.6</td>
</tr>
<tr>
<td>B2: Probable human carcinogen (sufficient animal data)</td>
<td>64</td>
<td>27.2</td>
</tr>
<tr>
<td>C: Possible human carcinogen (animal data inadequate for stronger classification)</td>
<td>53</td>
<td>22.6</td>
</tr>
<tr>
<td>D: Unclassifiable (animal data inadequate for stronger classification)</td>
<td>58</td>
<td>24.7</td>
</tr>
<tr>
<td>E: Probable human non-carcinogen (sufficient animal data)</td>
<td>3</td>
<td>1.3</td>
</tr>
<tr>
<td>TOTAL</td>
<td>235</td>
<td></td>
</tr>
</tbody>
</table>

160 chemicals lacking in human data had received a human carcinogenicity assessment primarily on the basis of their animal data.

1 Chi-squared and two-tailed p values were derived from the online statistical calculators available at www.graphpad.com/quickcalcs/index.cfm.
2 Confidence interval derived via the modified Wald method described by Agresti et al. (1998) as being more accurate than the so-called “exact” method commonly used.
3 Chi-squared analysis does not allow comparison when one category lacks any data, hence acrylonitrile, assessed as the only possible human carcinogen by IARC, but as a probable human carcinogen (B1) by the EPA, was excluded, yielding a more conservative result.
4 To allow chi-squared analysis, methacrylate, assessed as unclassifiable by IARC, but as the only probable human non-carcinogen by the EPA, was excluded, yielding a more conservative result.

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data but having animal data, the EPA considered the animal data inadequate to support the substantially useful classifications of probable human carcinogen or probable human non-carcinogen in the majority of cases (93/160; 58.1%, 95% CI: 50.4-65.5\(^2\)).

**Comparison of EPA and IARC human carcinogenicity classifications**

Of those 177 chemicals considered by the EPA to possess human or animal data (A, B1, B2, C, D with animal data, or E), 128 were also assessed by the IARC. Of these, 17 were considered by the EPA to possess at least limited human data (A or B1), and the remaining 111 EPA carcinogenicity classifications were primarily reliant on animal data.

For those 17 chemicals considered by the EPA to possess at least limited human data, overall EPA classifications were not found to differ significantly from those predicted by IARC classifications (\(p = 0.5896, \chi^2 = 0.291, 1\) df, tab. 2\(^3\)).

However, for those 111 chemicals considered by the EPA to lack even limited human data, but to possess animal data, EPA and IARC classifications were very significantly different overall (\(p < 0.0001, \chi^2 = 215.548, 2\) df; fig. 1\(^4\)).

The EPA was much likelier than the IARC to assign carcinogenicity classifications indicative of greater human hazard. The EPA classified 60 chemicals as probable human carcinogens and 51 in all other categories, which was very significantly different from the IARC figures of 12 and 99, respectively (\(p < 0.0001, \chi^2 = 215.273, 1\) df). Similar disparities were found for possible human carcinogens (\(\chi^2 = 19.771, 1\) df, \(p < 0.0001\)) and unclassifiable chemicals (\(p < 0.0001, \chi^2 = 24.378, 1\) df).

**Comparison of individual classifications**

Comparison of the individual classifications of these 111 chemicals revealed that 67 (60.4%) were assigned an EPA carcinogenicity classification indicative of greater human hazard, 38 (34.2%) were assigned an equivalent classification, and 6 (5.4%) were assigned a classification indicative of lesser human hazard than the corresponding IARC classification of the same chemical.

**Discussion**

Based on EPA figures alone, the predictivity of animal carcinogenicity data for human hazard, and hence its utility in deriving substantially useful human carcinogenicity classifications, is
clearly poor. Of those 160 IRIS chemicals lacking even limited human data but possessing animal data, the EPA considered the animal data inadequate to support substantially useful human carcinogenicity classifications in the majority (58%) of cases.

However, IARC assessments of the same chemicals reveal that the human utility of animal carcinogenicity data is probably even lower than indicated by EPA figures. EPA and IARC carcinogenicity classifications were similar only for those chemicals with human data. For those with only animal data, the EPA was much likelier than the IARC to assign carcinogenicity classifications indicative of greater human hazard.

Given that the IARC is recognised as a leading international authority on human carcinogenicity classifications (Tomatis et al., 1993; IARC undated), the very significant differences in classifications of identical chemicals between the IARC and the EPA indicate that:
- (i) in the absence of significant human data the EPA is over-reliant on animal carcinogenicity data,
- (ii) as a result, the EPA tends to over-predict carcinogenic risk, and
- (iii) the true human predictivity for human carcinogenicity of animal data is even poorer than indicated by EPA figures alone.

EPA human carcinogenicity classifications appear to be less scientifically-based than those of the IARC, due to: 1) the varying depth of EPA assessments, due to resource constraints; 2) the less rigorous standards required of data incorporated into EPA assessments; and, in particular, 3) EPA public health-protective policy, which errs on the side of caution by assuming that tumours in animals are indicative of human carcinogenicity (Knight et al. 2006).

Our findings corroborate those of previous investigators. In response to a 2000 Congressional directive, the EPA undertook an evaluation of the data variability and uncertainty within its IRIS assessments. A representative sample of 16 IRIS assessments was subjected to in-depth evaluation by a panel of six independent experts, who concluded that despite being advertised as quantitative science-based classifications, some were, in fact, more grounded in EPA policy favouring classifications indicative of greater human risk (Hogan, 2000).

EPA carcinogenicity assessments may be no more suspect than those of other U.S. regulatory agencies, however. In their survey of 350 representative chemicals, Viscusi and Hakes (1998) found that the carcinogenicity assessments of other U.S. regulatory authorities, particularly the Food and Drug Administration and the Occupational Safety and Health Administration, are even less reflective of actual human risk than those of the EPA. Poor human predictivity of animal carcinogenicity studies was also demonstrated by Tomatis and Wilbourn (1993) and Haseman (2000), and further described by Rall (2000), Ashby and Purchase (1993), Fung et al. (1995) and Ennever and Lave (2003).

Conclusions
By 1998, only about 2,000 (2.7%) of the 75,000 industrial chemicals in use and listed in the EPA’s Toxic Substances Control Act inventory had been tested for carcinogenicity (Epstein, 1998). The cost of testing these 2.7% of industrial chemicals was millions of animal lives (Monro et al., 1998; Gold et al., 1999), millions of skilled personnel hours (Gold et al., 1999), and hundreds of millions of dollars (Greek et al., 2000; Stephens et al., 1998).

The most important use of the animal data thus derived is in the regulation of human exposures to potential carcinogens by governmental agencies such as the EPA. However, our results demonstrate that the human predictivity of animal carcinogenicity data was inadequate for the EPA to derive substantially useful human carcinogenicity classifications for the majority (58.1%) of chemicals of greatest public health concern.

Profound differences in human carcinogenicity classifications of identical chemicals between the EPA and the IARC reveal an over-reliance on animal carcinogenicity data by the EPA. The result is that the EPA over-predicts carcinogenic risk. Hence the true human predictivity of animal carcinogenicity data is even poorer than indicated by EPA figures alone.

The sensitivity of the traditional rodent bioassay in detecting human carcinogens for some sex-species combinations is not in question. However, its very poor human specificity severely limits its utility for identifying human carcinogens, and its subsequent use in regulating exposures to them. The implementation by regulatory authorities of alternative assays with superior human predictivity is clearly necessary.

References
The modified Wald method is also described at http://www.graphpad.com/quickcalcs/ConfInterval2.cfm.

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Correspondence to
Andrew Knight BSc., BVMS, Cert AW, MRCVS
Director
Animal Consultants International
91 Vanbrugh Court, Wincott St.
London SE11 4NR, UK
e-mail: info@animalconsultants.org
Internet: www.AnimalConsultants.org
Detection of Non-Genotoxic Carcinogens Using Ras-Transfected Bhas 42 Cells

Makoto Umeda
Hatano Research Institute, Food and Drug Safety Center, Kanagawa, Japan

Summary
The cell transformation test employing Bhas 42 cells (v-Ha-ras-transfected BALB/c 3T3 cells) was shown to be a sensitive screening method for the detection of chemicals with different mechanisms of transforming potential. Because of the simplicity of the procedure, it is anticipated that further investigations on Bhas assays will open ways of not only detecting non-genotoxic carcinogens but also of elucidating mechanisms of carcinogenesis.

Keywords: cell transformation assay, Bhas 42 cells, detection of initiating and promoting activities

Introduction

In vitro cell transformation tests can simulate the process of two-stage animal carcinogenesis, and were expected to be an efficient alternative method of animal tumourigenicity tests (IARC/NCI/EPA Working Group, 1985). As the formation of transformed foci is the consequence of the complex process of cell malignisation, the tests can be anticipated to be useful for the detection of not only tumour initiators and promoters, but also of non-genotoxic carcinogens. In spite of this expectation, none of the in vitro cell transformation tests have been accepted as routine screening methods, because the tests are thought to be laborious and time-consuming compared with current routine genotoxicity tests.

Ohmori et al. have developed an in vitro cell transformation assay for tumour promoters using Bhas 42 cells (Ohmori et al., 2004). The cells are v-Ha-ras-transfected BALB/c 3T3 cells and were considered initiated (Sasaki et al., 1988 and 1990). Recently, in addition to the promotion assay, we found that Bhas cells could be transformed after treatment with initiators, and thus developed an assay method for the evaluation of initiating activity using these cells (Asada et al., 2005). The Bhas transformation assay is a sensitive method and has many advantages, such as shortened experimental time, use of less material and simplicity of the procedure.

Here, we introduce the method for detecting initiating and promoting activities using Bhas 42 cells, and report results obtained by the method, some of which will appear elsewhere (Asada et al., in press; Ohmori et al., 2005).

Materials and methods

Bhas 42 cells were routinely cultured in Minimum Essential Medium (MEM) supplemented with 10% foetal bovine serum (FBS) (M10F). For transformation assays, Dulbecco’s modified Eagle’s medium/Ham’s F12 (DMEM/F12) supplemented with 5% FBS (DF5F) was used. DF212F medium used for the growth assay consisted of DMEM/F12 supplemented with 2 µg/ml insulin and 2% FBS.

Experimental procedures are depicted in fig. 1, in which the original BALB/c 3T3 cell transformation assay was included for comparison. In the Bhas 1st-stage assay for the detection of initiating activity, 4x10³ cells in 2 ml were seeded into 6-well plates. Test chemicals were added on day 1, and from day 4 the cells were cultured in fresh DF5F medium until day 21. In the Bhas 2nd-stage assay for the detection of promoting activity, 1.4x10⁴ cells were inoculated into wells, and test chemicals were added to the medium on days 4, 7 and 11 when the medium was changed. Cultures were continued until day 21. After fixation and staining, transformed foci were scored under a stereomicroscope. Transformed foci were judged by morphological characteristics: deep basophilicity, dense multilayering of cells, random orientation of cells at the edge of foci, and more than 50 cells within a focus.

Cell growth was assayed using the crystal violet staining method (Saotome et al., 1989). Cells cultured in 24-well plates were fixed with formalin and stained with crystal violet (CV) solution. CV was extracted from the stained cells in each well, and optical density of extracted CV was measured at 540 nm. The results were expressed as percentage of absorbance compared to the solvent control culture.

Results and discussion

Detection of both initiating and promoting activities and tests on PAHs

Typical tumour initiators were examined in Bhas 1st-stage and 2nd-stage assays. Fig. 2 shows results of treatment with typical tumour initiators, N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and 3-methylcholanthrene (MCA). Induction of transformed foci was observed in the 1st-stage assay, but not in the 2nd-stage assay. In contrast, typical tumour promoters, 12-O-
Fig. 1: In vitro cell transformation protocols using BALB/c 3T3 cells and Bhas 42 cells

Fig. 2: Bhas 1st-stage and 2nd-stage transformation assays on typical tumour initiators

Fig. 3: Bhas 1st-stage and 2nd-stage transformation assays on typical tumour promoters
tetradecanoylphorbol-13-acetate (TPA) and lithocholic acid (LCA), were negative in the 1st-stage assay, but positive in the 2nd-stage assay (see fig. 3).

Fig. 4 shows results of repeated treatment with MCA (100 ng/ml) and/or TPA (50 ng/ml). In this experiment, Bhas 42 cells seeded at 4x10^3 cells/well were used. Repeated treatment with MCA in the growth phase (1st-stage) increased the number of transformed foci (schedule H), but treatment in the stationary phase (2nd-stage) (schedule D) did not. The opposite situation was observed upon treatment with TPA (schedule E and I). Some tumour initiators are considered to have tumour-promoting activity owing to the experimental observation that repeated application at subtumourigenic doses can induce tumours in mouse skin without any post-treatment with promoter (Berenblum, 1941). Our observation that MCA was effective only when cells were in a dividing phase and TPA was active when cells were in a stationary phase, suggests that the action of tumour initiators is completely different from that of tumour promoters.

Results from both assays on various polycyclic aromatic hydrocarbons (PAHs) and some others are summarised in table 1. Chemicals showing only initiating activity, promoting activity, and both positive and both negative activities are listed. Thus, Bhas cells were reactive to PAHs. The results showing that there are various kinds of PAHs with initiating and/or promoting activities suggest the hazardous nature of mixtures of PAHs such as particulate matter in polluted air, etc.

**Collaborative work on Bhas 2nd-stage assay**

In collaboration with 14 laboratories in Japan, an inter-laboratory study was conducted in order to validate transferability and applicability of the Bhas 2nd-stage assay (Ohmori et al., 2005). Detailed results of this study were presented in the poster session of this meeting. Briefly, in addition to TPA and LCA, 12

### Tab. 1: Summary of Bhas transformation assays on polycyclic aromatic hydrocarbons and typical initiators and promoters

<table>
<thead>
<tr>
<th>Result of 1st stage assay</th>
<th>Result of 2nd-stage assay</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Benz[a]anthracene</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Benz[b]anthracene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Perylene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12-O-Tetradecanoylphorbol-13-acetate</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>Anthracene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acenaphthylene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Coronene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9,10-Diphenylanthracene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Naphthalene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phenanthrene</td>
<td></td>
</tr>
</tbody>
</table>

### Tab. 2: Results of collaborative studies of Bhas 2nd-stage assay on 14 chemicals

<table>
<thead>
<tr>
<th>Chemical</th>
<th>No. of Lab. examined</th>
<th>Judgment</th>
<th>Type of promoter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>Equivocal</td>
</tr>
<tr>
<td>TPA</td>
<td>12</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>LCA</td>
<td>12</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Anthraine</td>
<td>4</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Catechol</td>
<td>4</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Insulin</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Mezerein</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>4</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>PDD</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>4α-Phorbol</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Progesterone</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Sodium saccharin</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>
chemicals were assayed under blinded conditions. Each chemical was examined in 4 laboratories. Results of 3 positive chemicals are representatively shown in fig. 5. Judgments obtained from the different laboratories were highly consistent (see tab. 2).

This study suggested that there are at least three types of chemicals inducing positive results in the 2nd-stage assay (see fig. 6). Those designated as T(TPA)-type induced extreme growth enhancement, and included TPA, mezerein, PDD and insulin. LCA and okadaic acid belonged to the L(LCA)-type category, in which transformed foci were induced at concentrations showing growth inhibition. In contrast, progesterone, catechol and sodium saccharin (M(miscellaneous)-type) induced foci at concentrations with little to slight growth inhibition. Categorisation of these types is important to decide on doses to

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**Fig. 5: Representative results in inter-laboratory collaborative study of Bhas 42 cell assay**

**Fig. 4: MCA and/or TPA treatment under various time schedules on Bhas 42 cells**

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**PDD**

**Okadaic acid**

**Progesterone**
be used in the transformation assay after the preliminary cell growth assay, as well as to elucidate the mechanisms of tumour promotion.

**Tests on various chemicals**

Various chemicals were tested, and the results are summarised in table 3. Known tumour initiators, mitomycin C and methyl methanesulphonate, were demonstrated to be positive in the 1st-stage assay and negative in the 2nd-stage assay. Metabolites suggested to be tumour promoters were tested in the 2nd-stage assay.

In relation to LCA, other tested cholic acids, cholic acid, deoxycholic acid and chenodeoxycholic acid, tested positive. Tryptophan metabolites were suggested to be tumour promoters of the bladder. 3-Hydroxykynurenine showed highly potent activity in inducing foci in the 2nd-stage assay.

Tests on non-genotoxic carcinogens revealed that 17β-estradiol was equivocal in the 1st-stage assay. Methapyrilene hydrochloride and zinc chloride were positive in the 2nd-stage assay. Diethylstilbestrol, phenobarbital and reserpine were negative at present.
The number of chemicals examined was limited, but the results demonstrated that some non-genotoxic carcinogens could be detected in the Bhas assays. Because of the simplicity of the procedure, it is anticipated that further investigations on Bhas cells will open ways of not only detecting non-genotoxic carcinogens but also elucidating mechanisms of carcinogenesis.

**Growth of various cell lines in different media**

Various cell lines were cultured in different media, and their growth patterns were compared (fig 7). The growth pattern of Bhas 42 cells was similar to that of BALB/c 3T3 cells in M10F and DF5F media. After both cell lines reached relatively low saturation, they entered into stationary phase, revealing non-transformed phenotype. In contrast, transformed derivatives of BALB/c 3T3 cells (4-1-1, 6-2-1 and BMT-50 clones) grew better than the non-transformed cells, reached high saturation density, and then detached from the culture surface. Thereafter, transformed cell clones resumed growth, but repeated detachment and regeneration. When saturation densities of transformed cell lines were compared to those of the non-transformed cell lines, the saturation densities differed 2- to 4-fold. The difference of this increase was greater in DF5F medium than M10F medium.

In general, growth of cell lines in DF212F medium was lower than in other media, and that of Bhas 42 was lowest. 2% FBS in the medium was not enough for the growth of Bhas 42 cells, even with the addition of 2 µg/ml insulin.

**References**


Ohmori, K., Umeda, M., Tanaka, N. et al. (2005). An inter-laboratory collaborative study by the non-genotoxic carcinogen study group in Japan, on a cell transformation assay for tumour promoters using Bhas 42 cells. *ATLA* 33, 619-639.


**Correspondence to**

Makoto Umeda
729-5 Ochiai
Hadano
Kanagawa Pref. 257-0025
Japan
e-mail: GZH04502@nifty.ne.jp

**Tab. 3: Results of transformation assays on various chemicals**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>1st-stage assay</th>
<th>2nd-stage assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitomysin C</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Methyl methanesulfonate</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Cholic acid</td>
<td>Not tested</td>
<td>+</td>
</tr>
<tr>
<td>Deoxycholic acid</td>
<td>Not tested</td>
<td>+</td>
</tr>
<tr>
<td>Chenodeoxycholic acid</td>
<td>Not tested</td>
<td>+</td>
</tr>
<tr>
<td>Kynurene</td>
<td>Not tested</td>
<td>+/-</td>
</tr>
<tr>
<td>3-Hydroxy kynurenine</td>
<td>Not tested</td>
<td>+</td>
</tr>
<tr>
<td>Anthranilic acid</td>
<td>Not tested</td>
<td>-</td>
</tr>
<tr>
<td>3-Hydroxy anthranilic acid</td>
<td>Not tested</td>
<td>-</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17β Estradiol</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>Methapyrilene hydrochloride</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Phenacetin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Reserpine</td>
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A Study of Connexin Expression in Monolayer Cultures of Primary Rat Hepatocytes

Mathieu Vinken1,2, Tamara Vanhaecke1, Peggy Papeleu1, Sarah Snykers1, Tom Henkens1, Greetje Elaut1, James Kevin Chipman2 and Vera Rogiers1

1Department of Toxicology, Vrije Universiteit Brussel (VUB), Brussels, Belgium, 2School of Biosciences, University of Birmingham, Birmingham, United Kingdom, 3Mathieu Vinken is a doctoral research fellow of the Fund for Scientific Research Flanders (FWO-Vlaanderen), Belgium

Summary

Dedifferentiation in primary cultures of adult rat hepatocytes was studied at the level of connexin expression. It was found that Cx32 protein levels gradually declined, whereas Cx26 production remained relatively stable during seven days of cultivation. Cx43, a connexin species produced by foetal hepatocytes but not by their adult counterparts, progressively appeared and was present both at the cell membrane surface and in the cytosol. Overall, this connexin switching process was associated with low gap junctional intercellular communication activity. These data provide new insights into hepatocyte dedifferentiation and aid in the optimisation of long-term primary hepatocyte cultures.

Keywords: primary hepatocyte, gap junctional intercellular communication, connexin

Introduction

Co-operation between hepatocytes is a prerequisite for normal liver functioning. In particular, direct communication via gap junctions is known to control several liver-specific functional traits including xenobiotic biotransformation (Hamilton et al., 2001; Shoda et al., 1999). Basically, this results from their capacity to mediate the exchange of small and hydrophilic molecules (e.g. Ca2+ and cAMP) between hepatocytes. This flux is called the gap junctional intercellular communication (GJIC). Gap junctions are composed of two connexons, in turn built up by six connexin (Cx) proteins. In liver, hepatocytes express both Cx32 and Cx26, whereas Cx43 is the only connexin species produced by non-parenchymal hepatic cells (Saez et al., 2003; Spray et al., 1994).

Primary hepatocyte cultures are valuable in vitro tools for pharmaco-toxicological testing of chemicals. A major shortcoming, however, includes the progressive deterioration of the hepatocyte-specific phenotype (Papeleu et al., 2005). This dedifferentiation process is also reflected at the level of GJIC. Indeed, GJIC between primary cultured hepatocytes rapidly declines, ultimately resulting in the loss of liver-specific functionality (Stoehr and Isom, 2003).

Here, we explore the mechanisms that underlie decreased GJIC between hepatocytes by studying connexin expression at the transcriptional, translational and activity level. These data are of interest for the development of new strategies to reduce dedifferentiation in primary hepatocyte cultures.

Materials and methods

Animals, hepatocyte isolation and cultivation

Procedures for the isolation and cultivation of rat hepatocytes were approved by the local ethical committee of the Vrije Universiteit Brussel. Hepatocytes (viability >80%) were isolated from adult male outbred (200-250 g) Sprague-Dawley rats (Charles River Laboratories, Belgium) by use of a two-step collagenase method (Papeleu et al., 2005), and cultivated in a monolayer configuration. The medium used was DMEM containing 0.5 IU/ml insulin, 7 ng/ml glucagon, 292 mg/ml L-glutamine, antibiotics (7.3 IU/ml benzyl penicillin, 292 mg/ml L-glutamine, antibiotics (7.3 IU/ml benzyl penicillin, 50 µg/ml kanamycin monosulphate, 10 µg/ml sodium ampicillin, 50 µg/ml streptomycin sulphate) and 10% v/v foetal bovine serum. After 4 h, the medium was removed and renewed with fresh medium supplemented with 25 µg/ml hydrocortisone hemisuccinate and 0.25 µg/ml amphotericin B. After 24 h, the medium was renewed daily with serum-free medium. Samples were taken on days 1, 4 and 7 of the cultivation time.

RNA extraction and RT-PCR

Cells were harvested from culture plates by scraping and washed twice with cold PBS. Total cellular RNA was extracted using the SV Total RNA isolation system (Promega, USA) according to the manufacturer’s instructions, and samples were subsequently subjected to DNase treatment (Ambion, USA). Reverse transcription of approximately 2 µg of total RNA was carried out using the iScript cDNA synthesis kit (Bio-Rad, Germany). For
PCR, 2 µg cDNA was amplified by means of the iTaq DNA polymerase kit (Bio-Rad, Germany). PCR primers used are listed in table 1. PCR conditions included, for Cx26: 40 cycles (1 min at 95°C, 1 min at 53.2°C, 1 min at 72°C); for Cx32: 35 cycles (1 min at 95°C, 1 min at 59.1°C, 1 min at 72°C); for Cx43: 40 cycles (1 min at 95°C, 1 min at 59.6°C, 1 min at 72°C); for GAPDH: 25 cycles (1 min at 95°C, 1 min at 52.7°C, 1 min at 72°C), each followed by final elongation at 72°C for 7 min. Samples were separated by electrophoresis on a 1.8% w/v agarose gel. Visualisation of the products was performed with ethidium bromide (0.005% v/v) under UV transillumination.

Preparation of cell lysates and immunoblotting
Cells were harvested from culture plates by scraping, washed with cold PBS, and lysed in modified Tween-20 buffer (Albrecht and Hansen, 1999). Protein concentrations were determined according to the Bradford procedure (Bradford, 1976), using a commercial kit (Bio-Rad, Germany) with bovine serum albumin as standard. Proteins (25 or 50 µg) were resolved on SDS-PAGE (7.5%, 10% or 12%) and blotted afterwards onto nitrocellulose membranes (Amersham Pharmacia Biotech, UK). Following blocking with fat-free milk, membranes were incubated with appropriate concentrations of primary antibodies (tab. 2) and HRP-labelled secondary antibodies (Dakocytomation, Denmark). Proteins were detected using the ECL detection system (Amersham Pharmacia Biotech, UK). Specificity of the primary antibodies was tested by means of positive controls, i.e. rat exocrine pancreatic tissue (10 µg protein) for Cx26 and Cx32, and rat heart homogenate (100 µg protein) for Cx43. Immunoreactivity of the Cx43 antibody in primary rat hepatocytes was further evaluated by incubating 7 day-old cell lysates overnight at 37°C with 30 IU alkaline phosphatase (AP) from calf intestine (Roche, Germany).

<table>
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<td>R: 5'-GAAGATGAGCTGTCAGGAC-3'</td>
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<tr>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
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</tr>
<tr>
<td></td>
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Immunocytochemistry
Hepatocytes were fixed with cold ethanol, permeabilised with Triton X-100, and blocked with fat-free milk. Following incubation with appropriate concentrations of primary antibodies (tab. 2) and FITC-labelled or TRITC-conjugated secondary antibodies (Zymed, USA), samples were mounted with DAPI-containing Vectashield (Vector Laboratories, USA). Detection of proteins was performed by fluorescence microscopy (Leica DMR/XA).

GJIC assay
GJIC was monitored by means of the scrape loading/dye transfer assay (el-Fouly et al., 1987) using 0.1% w/v Lucifer Yellow CH (Sigma, USA). Dye transfer was evaluated by fluorescence microscopy (Leica DMR/XA).

Results
Specificity of the primary antibodies
Connexin nomenclature is based upon molecular weight, as predicted by cDNA sequencing (Saez et al., 2003). The Cx26 antibody indeed recognised a 26 kDa protein in rat hepatocytes and in rat exocrine pancreatic cells (fig. 1A). In both cell types, however, the Cx32 antibody detected a protein of approximately 27 kDa (fig. 1A). Similar results have also been obtained by others and this finding might be explained by the occurrence of partial proteolysis of the native Cx32 protein during protein extraction, yielding a smaller (i.e. 27 kDa) protein (Kumar and Gilula, 1986; Paul, 1986).

Unlike for most other connexins, phosphorylation of Cx43 can be detected by SDS-PAGE analysis, appearing as a multiband signal (VanSlyke and Musil, 2000). The Cx43 antibody used here was claimed to specifically recognise the unphosphorylated Cx43 form, located at 41 kDa. When applied to rat heart tissue, a natural source of Cx43, the antibody indeed displayed single band immunoreactivity as shown in figure 1B and by others (Nagy et al., 1997). In 7 day-old primary rat hepatocytes, however, this antibody detected two closely related proteins at 41 kDa and 43 kDa (fig. 1B). Therefore, day 7 samples were treated overnight with AP. Upon subsequent immunoblot analysis, the upper band (43 kDa) became less intense (fig. 1B), suggesting that this signal corresponds with a phosphorylated Cx43 form. This finding is in agreement with the experiments of Cruciani and Mikalsen. In their model, a fibroblast cell line system, this antibody detected several phosphorylated Cx43 forms (Cruciani and Mikalsen, 1999). Thus, the 13-8300 antibody is

<table>
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able to recognise at least one phosphorylated Cx43 form, next to its unphosphorylated counterpart, depending on the experimental model used.

**Expression of connexins in primary cultures of rat hepatocytes**

Connexin expression in primary cultured rat hepatocytes was studied both at the translational and the transcriptional level. In immunoblot experiments, rat liver homogenate (LH) was included in order to have reference data for connexin expression in hepatic tissue *in vivo*.

As shown in figure 2, Cx32 protein levels decreased in primary cultures of rat hepatocytes. Cx26 protein expression, however, remained relatively stable during the whole cultivation period. In contrast, Cx43, which is not expressed by adult hepatocytes *in vivo*, progressively appeared in primary cultured rat hepatocytes. These alterations in connexin expression were also observed at the mRNA level, albeit to a lesser extent (fig. 3).

**Localisation of connexins in primary cultures of rat hepatocytes**

Immunocytochemistry was used to study connexin localisation during cultivation of hepatocytes (fig. 4). On day 1, Cx32 and Cx26 were mainly located in the cytosol of hepatocytes. Most likely, this is an inevitable consequence of the isolation procedure. Membrane localisation of both connexins was restored on day 4. Weak signals were observed on day 7, especially in case of Cx32. In contrast, Cx43 staining became evident on day 4, and was clearly present on day 7. Cx43 immunoreactivity was observed both at the cell membrane surface and in the cytosol of hepatocytes (fig. 4).

**GJIC in primary cultures of rat hepatocytes**

GJIC was evaluated by using the scrape loading/dye transfer method (fig. 5). On day 1, dye transfer between hepatocytes was limited. This finding corresponds with the cytosolic localisation of connexins observed at that time point. Dye transfer was slightly increased on day 4, but remained limited on day 7 (fig. 5).

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**Fig. 1 A**

![Image of immunoblot results showing Cx26 and Cx32 expression levels in rat hepatocytes and liver homogenate.](image)

**Fig. 1 B**

![Image of immunoblot results showing Cx43 expression levels in rat hepatocytes and liver homogenate with and without alkaline phosphatase treatment.](image)

**Fig. 1 A and B: Specificity of the primary antibodies.**

(A) Immunoreactivity of the 71-0500 (upper panel) and C3470 (lower panel) antibodies in 1 day-old primary rat hepatocytes (HP) and in rat exocrine pancreatic cells (P). (B) Upper panel: immunoreactivity of the 13-8300 antibody in 7 day-old primary rat hepatocytes (HP) and in rat heart homogenate (H). Lower panel: immunoreactivity of the 13-8300 antibody in 7 day-old primary rat hepatocytes after overnight incubation at 37°C in the absence (-) or presence (+) of 30 IU alkaline phosphatase (AP).

**Fig. 2: Connexin protein levels in primary cultures of rat hepatocytes.**

Samples were taken on days 1, 4 and 7 (D1, D4, D7) of the cultivation time and subjected to immunoblot analysis. Semi-quantification of immunoblot analyses was performed by measuring band intensities using laser densitometry. Results are expressed as fold of liver homogenate (LH) and are mean values ± standard deviation of three independent experiments.
Conclusions and perspectives

In this study, it was investigated whether the deterioration of GJIC in primary cultures of hepatocytes, known to underlie dedifferentiation, is associated with alterations in connexin expression. It was found that Cx32 protein levels rapidly decline, whereas the Cx26 production remained relatively stable during seven days of cultivation. In agreement with previous reports (Stutenkemper et al., 1992; Willecke and Haubrich, 1996), we showed that Cx43, a connexin species produced by foetal hepatocytes but not by their adult counterparts, progressively appeared in culture. Cx43 was present both at the cell membrane and in the cytosol of hepatocytes, thereby questioning its functional relevance. In fact, the molecular basis of Cx43 re-expression in primary cultured hepatocytes remains obscure. Possibly, this results from altered cis/trans regulation of its gene expression. Upon isolation of hepatocytes, c-fos and c-jun productions are induced (Etienne et al., 1988; Loyer et al., 1996). These proto-oncogenes dimerise to form the transcription factor activator protein-1 (AP-1), which is known to control Cx43 expression (Echetebu et al., 1999). In rat myometrium, AP-1 has been shown to induce Cx43 expression under stress conditions (Lefebvre et al., 1995). Although no solid scientific data are presently available, this scenario might also hold for isolated primary hepatocytes in culture.

Several groups have focused on the re-establishment of GJIC in primary hepatocyte cultures. Three strategies are currently followed to restore hepatocyte GJIC in vitro, namely (i) the introduction of an extracellular matrix, by cultivating hepatocytes on one layer or between two layers of extracellular matrix proteins (Spray et al., 1987), (ii) the establishment of cell-cell contacts, by co-cultivating hepatocytes with another cell type (Mesnil et al., 1993), and (iii) the addition of GJIC-promoting molecules to the medium of cultured hepatocytes (Yoshizawa et al., 1997).

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Fig. 3: Connexin gene expression in primary cultures of rat hepatocytes.
Samples were taken on days 1, 4 and 7 (D1, D4, D7) of the cultivation time and subjected to RT-PCR analysis as described in "Materials and methods".

Fig. 4: Connexin protein localisation in primary cultures of rat hepatocytes.
Samples were taken on days 1, 4 and 7 (D1, D4, D7) of the cultivation time and subjected to immunocytochemistry analysis.

Fig. 5: GJIC in primary cultures of rat hepatocytes.
Samples were taken on days 1, 4 and 7 (D1, D4, D7) of the cultivation time and subjected to scrape loading/dye transfer analysis.
Our group is currently exploring the use of histone deacetylase inhibitors as medium supplements for primary hepatocytes. With respect to gap junctions, we have shown that trichostatin A, a prototype histone deacetylase inhibitor, promotes Cx32 expression and, consequently, GJIC (Vinken et al., in press). Further efforts to regain GJIC and thus to reduce dedifferentiation in primary cultures of hepatocytes should be strongly encouraged, as they contribute to the optimisation of liver-based in vitro models for long-term pharmaco-toxicological testing.

References


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Correspondence to

Mathieu Vinken
Vrije Universiteit Brussel (VUB)
Department of Toxicology
Laarbeeklaan 103
1090 Brussels, Belgium
e-mail: mvinken@vub.ac.be