Bhas42 cell transformation assay as a predictor of carcinogenicity

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Abstract
The Bhas-system was established by using the reference carcinogen 3-methylcholanthrene as an initiator and TPA and Insulin as promoters. Increasing concentrations led to a dose-dependent increase in transformed foci. By selective trypsinization separate cultures could be established from foci cells and from normal cells. Micronucleus frequencies turned out to be 1.7 fold higher in cultures of transformed cells. More surprisingly, no significant differences in mitotic rates and cell cycle distribution between cultures of transformed and normal cells exist. Nevertheless, parallel staining of microfilaments and nuclei clearly displayed a dense and multilayered piling-up of cells in foci. Thus alterations in adhesion protein profiles and enzyme activities modulating cellular adhesion and migration seem to be involved in the process of focus formation. These may be promising candidates for the identification of molecular markers leading to a more objective scoring of foci and a greater acceptance of cell transformation tests in general.

Keywords: cell transformation, in vitro carcinogenicity, Bhas cells, biochemical markers

Introduction
Carcinogenesis is a multistep process which involves sequential genetic alterations in a single target cell ultimately leading to malignant transformation and tumour development. Carcinogen-induced alterations in DNA play a causal role. In vitro cell transformation tests using SHE, BALB/c 3T3 or C3H10T1/2 cells simulate the process of animal two-stage carcinogenesis. These tests may be well suited for the in vitro detection of a carcinogenic potential of test compounds in risk assessment. A limitation of these methods however is the time required for focus formation as a morphological indicator of transformation.

Thus, Sasaki et al. (1988) developed the cell line Bhas42 by transfecting BALB/c 3T3 cells with the v-Ha-ras oncogene. Using these cells, transformed foci can be efficiently induced by treatment with initiating and promoting agent.

Material and methods
Bhas42 cells were routinely cultured in MEM supplemented with 10% FCS in a humidified 5% CO₂ incubator at 37 °C. The cells were subcultured using 0.25% trypsin before reaching confluence. For transformation assays, Bhas42 cells were cultured in DMEM/F12 supplemented with 5% FCS. The test compounds used for establishing the assay were 3-methylcholanthrene (3-MCA) and 2-acetylaminofluorene (2-AAF) as carcinogenic and m-anisidine as a non-carcinogenic compound. The following concentrations were used for treatment of the cultures: 0.1/0.4/1.1/3.7/11.2 µM 3-MCA; 1.4/4.2/12.4/37.3/112 µM 2-AAF; 20/60/180/541/1624 µM m-anisidine. The cell growth was measured by standard crystal violet (CV) adsorption method and the concentrations in the main experiment were selected accordingly. Additionally, the cell growth assay was performed in parallel with every transformation assay.

For the initiation assay Bhas42 cells were adjusted to 2 x 10³ cells/mL in MEM and seeded into each well of 6-well microplates in 2 mL amounts (day 0). Six wells were prepared for each test concentration. After a 24 h cultivation period, medium was replaced with fresh medium containing the test chemical.

After a 24 h cultivation, medium was replaced with fresh medium containing the test chemical. Culture medium was replaced with DMEM/F12 without test chemical on day 3. Thereafter, culture medium was changed twice a week with fresh DMEM/F12 without test chemical. On day 24, the cells were fixed with methanol and stained with 5% Giemsa solution. Transformed foci were judged from
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.

The investigation of a biomarker, the butyrylcholinesterase (BChE) activity of the foci formed was determined by histochemical staining according to Layer and Willbold (1995). Cells were seeded and treated with 3 µM 3-MCA as described before and stained with a solution containing a coloured substrate (butyrylthiocholiniodide). Bhas42 cells with increased enzyme activity showed a dense brown staining.

For statistical analysis data were compared using one-way ANOVA. The significance level chosen for all statistical analyses was p > 0.05, if not indicated otherwise.

Results

The Bhas42 cell transformation assay was established by using the reference carcinogen 3-MCA. Treatment with 3-MCA led to a dose-dependent increase in transformed foci type III (Fig. 3), being in line with published data from rodent carcinogenicity tests (Table 1). The carcinogen 2-AAF was tested positive in a dose-dependent manner (Fig. 4), and the non-carcinogenic m-anisidine showed negative results (Fig. 5). However, no external metabolic activation system such as S9-mix was employed in these tests. Investigations into the metabolic competence of Bhas42 cells revealed no detectable basal cytochrome P450IA activity (data not shown).

By selective trypsinization separate cultures could be established from foci cells and from normal cells. Surprisingly, no significant differences in mitotic rates, cell cycle distribution and population doubling times between cultures of transformed and normal cells exist. Nevertheless, parallel staining of microfilaments and nuclei clearly displayed a dense and multilayered piling-up of cells in foci (data not shown).

Bhas42 cells growing as a monolayer of non-transformed cells showed no increased activity of BChE, whereas in altered cells of foci type II little BChE activity could be detected. Morphologically

Fig. 1: Type III foci after treatment with MCA on day 24 of the initiation assay

Fig. 2: Results of two independent Bhas42 cell transformation test with 2-AAF; Data are shown as means ± STD of two independent experiments; employing six-fold replicates each; * indicates significant difference p ≤ 0.05; dashed line represents negative control

Fig. 3: Results of three independent Bhas42 cell transformation tests with 3-MCA; Data are shown as means ± STD of three independent experiments, employing sixfold replicates each; * indicates significant difference, p ≤ 0.05; dashed line represents negative control

Fig. 4: Results of two independent Bhas42 cell transformation tests with m-anisidine; Data are shown as means ± STD of two independent experiments; employing sixfold replicates each; dashed line represents negative control

Fig. 5: Results of two independent Bhas42 cell transformation test with 2-AAF; Data are shown as means ± STD of two independent experiments; employing six-fold replicates each; * indicates significant difference p ≤ 0.05; dashed line represents negative control
Conclusion

Using 3-methylcholanthrene and 2-acetylaminofluorene as carcinogenic test compounds, a reproducible and strictly dose dependent increase in transformed foci (type III) was obtained. The non-carcinogenic compound m-anisidine showed a negative result in the cell transformation test.

Foci formation does not primarily depend on an increase in proliferation.

Alterations in adhesion protein profiles or enzyme activities modulating cellular adhesion and migration seem to be involved in the process of focus formation. These may be promising candidates for the identification of molecular markers leading to a more objective scoring of foci and a greater acceptance of cell transformation tests in general.

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References


Table 1: Compounds and the results from in vivo and in vitro testing for evaluation of their carcinogenic potential

<table>
<thead>
<tr>
<th>Test compound</th>
<th>Lifetime Rodent Bioassay</th>
<th>SHE assay</th>
<th>Balb 3T3 assay</th>
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<tbody>
<tr>
<td>2-acetylaminofluorene</td>
<td>Engelhardt, 2004</td>
<td>- Engelhardt, 2004</td>
<td>Not tested</td>
</tr>
<tr>
<td>m-anisidine</td>
<td>Not tested</td>
<td>- Engelhardt, 2004</td>
<td>Not tested</td>
</tr>
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Fig. 2: Histochemical staining of BChE activity in Bhas24 cells;
A: monolayer of non-transformed cells
B: Type II focus
C: Type III focus