British Journal of Cancer (1996) 74, (Suppl. XXVII) S136–S139
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Hypoxia-induced tetraploidisation of a diploid human melanoma cell line *in vitro*

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Summary Many human tumours are hyperdiploid, particularly in advanced stages of growth. The purpose of the present work was to investigate whether exposure to hypoxia followed by reoxygenation might induce hyperploidisation of diploid human tumour cells *in vitro*. The investigation was performed by using the diploid melanoma cell line BEX-c (median chromosome number, 46; DNA index, 1.10±0.04) as test line and the hyperdiploid melanoma cell line SAX-c (median chromosome number, 61; DNA index, 1.42±0.03) as control line. Cell cultures kept in glass dishes in air-tight steel chambers were exposed to hypoxia (O_2 concentrations <10 p.p.m. or <100 p.p.m.) at 37°C for 24 h. DNA content was measured by flow cytometry. Metaphase spreads banded with trypsin–Versene–Giemsa were examined to determine the number of chromosomes per cell. An electronic particle counter was used to measure cell volume. The expression of p53 and pRb was studied by Western blot analysis. Transient exposure to hypoxia was found to induce a doubling of the number of chromosomes in BEX-c but not in SAX-c. The fraction of the BEX-c metaphase spreads with 92 chromosomes was approximately 10% at 18 h after reoxygenation, decreased to approximately 2% at 7 days after reoxygenation and then increased gradually with time. The whole cell population became tetraploid within 25 weeks. BEX-c and SAX-c behaved differently during the 24 h hypoxia exposure. Cell volume and fraction of cells in G_2 + M increased with time in BEX-c but remained essentially unchanged in SAX-c. On the other hand, the expression of p53 and pRb was similar for the two lines; hypoxia induced increased expression of p53 and hypophosphorylation of pRb.

Keywords: hypoxia; melanoma; suppressor genes; tetraploidisation

Some experimental tumours implanted in transparent chambers show transient perfusion at the microvascular level, i.e. consecutive periods of non-perfusion and perfusion occurring in individual vessels or small groups of neighbouring vessels (Intaglietta *et al*., 1977). This observation led to the suggestion that malignant tissue might contain regions of acutely hypoxic cells (Brown, 1979), in addition to regions of chronically hypoxic cells (Thomlinson and Gray, 1955). Acute hypoxia promotes tumour progression and causes tumour treatment resistance (Hill, 1990). Thus, tumour cells subjected to acute hypoxia and reoxygenation might show increased metastatic potential (Young *et al*., 1988) and increased resistance to some chemotherapeutic agents (Luk *et al*., 1990). Moreover, exposure of tumour cells to transient hypoxia might induce cell subpopulations showing increased DNA content and gene expression (Rice *et al*., 1986).

Flow cytometric studies have shown that many human tumours are hyperdiploid, particularly in advanced stages of growth. Patients with hyperdiploid tumours usually have a poorer prognosis than patients with diploid tumours (Merkel and McGuire, 1990). The observation that hypoxia followed by reoxygenation might induce tetraploidisation of diploid human melanoma cells *in vitro* is reported in the present communication.

Materials and methods

Cell lines

Two human melanoma cell lines (BEX-c and SAX-c) were included in the study (Rofstad *et al*., 1991). BEX-c is diploid with a median chromosome number of 46 and a DNA index of 1.10±0.04 and was used as test line. SAX-c is hyperdiploid with a median chromosome number of 61 and a DNA index of 1.42±0.03 and was used as control line. The cell lines were maintained in RPMI-1640 medium (25 mM Hepes and L-glutamine) supplemented with 13% fetal calf serum, 250 mg l^{-1} penicillin and 50 mg l^{-1} streptomycin. Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air and subcultured by trypsinisation (0.05% trypsin/0.02% EDTA solution).

Exposure to hypoxia

Exponentially growing cell cultures plated in glass dishes were exposed to hypoxia (O_2 concentrations of < 10 p.p.m. or <100 p.p.m.) at 37°C for 24 h (Sanna and Rofstad, 1994). The glass dishes were placed in air-tight steel chambers which were flushed with a humidified, highly purified gas mixture consisting of 95% N_2 and 5% CO_2 at a flow rate of 5 l min^{-1}. The culture medium used during the hypoxia exposure was supplemented with 0.022 mg l^{-1} $NaHCO_3$. The pH in the medium at the end of the exposure was within the range of 7.3–7.5.

Cell clonogenicity assay

The clonogenicity of cells exposed to hypoxia was measured by seeding cells in 25 cm^2 tissue culture flasks containing 1×10^5 irradiated (30 Gy) feeder cells (Rofstad *et al*., 1991). Colonies developed within 7–10 days. They were fixed in 100% ethanol, stained with bromothymole blue and scored by using a stereomicroscope.

Flow cytometry

An Argus Skatron flow cytometer, equipped with an arch lamp as excitation source, was used for measurements of DNA histograms. Suspensions of clean cell nuclei were prepared from single-cell suspensions and stained with propidium iodide according to the detergent–trypsin method (Vindeløv *et al*., 1983). Excitation of propidium iodide was accomplished by using the 546 nm mercury line. Fluorescence was detected at wavelengths above 590 nm. Chicken and trout red blood cells were used as internal reference standards. Histograms were analysed mathematically to determine the distribution of cells in the cell cycle (Dean and Jett, 1974).

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Measurement of cell volume

Cell volume distributions were measured by using an electronic particle counter interfaced to a Coulter Channelyzer pulse-height analyser. The instrument was calibrated by means of monodisperse, electrically non-conductive microbeads.

Chromosome analysis

Cell cultures in exponential growth were exposed to Colcemid (2×10^{-4} $\mu g\ l^{-1}$) for 3 h. The cells were spread on glass slides after trypsinisation, hypotonic treatment in 0.075 M KCl for 8 min and fixation in methanol–glacial acetic acid. Metaphase spreads were banded with trypsin–Versene–Giemsa (Wang and Fedoroff, 1972). Photomicrographs were taken for chromosome analysis.

Western blot analysis

Cells were washed in phosphate-buffered saline and boiled in sample buffer for 5 min (Laemmli, 1970). The proteins were separated by electrophoresis in 10% sodium dodecyl sulphate–polyacrylamide gels (Laemmli, 1970) and blotted to polyvinylidene difluoride transfer membranes (Towbin *et al*., 1979). They were stained with anti-p53 (PAb1801, Oncogene Science) or anti-pRb (PMG3–245, Pharmingen) monoclonal antibodies by using a biotin–streptavidin alkaline phosphatase staining procedure.

Statistical analysis

Statistical comparisons of data were performed by non-parametric analysis using the Mann–Whitney *U*-test. A significance criterion of $P<0.05$ was used.

Results

Flow cytometric studies of BEX-c showed that a population of cells blocked in G_2 or M during the hypoxia treatment initiated DNA synthesis rather than completing mitosis after reoxygenation. Analysis of DNA histograms showed that this new cell population was asynchronous. Cells with DNA content corresponding to that of tetraploid G_2 + M cells appeared in the DNA histograms for the first time at 16–18 h after reoxygenation. The fraction of cells with larger DNA content than that of diploid G_2 + M cells increased gradually with time, reached a peak at 18 h after reoxygenation and then decreased to a stable level of 4–6% (Figure 1). In contrast, SAX-c did not develop a similar cell subpopulation with increased DNA content following exposure to hypoxia and reoxygenation (Figure 1).

Chromosome analysis revealed that approximately 10% of the BEX-c metaphase spreads showed 92 chromosomes at 18 h after reoxygenation. Homologous chromosomes were grouped in pairs (diplochromosomes) in approximately 70% of these metaphase spreads. In the remaining 30%, homologous chromosomes were clearly separated from each other and distributed randomly. The fraction of metaphase spreads with 92 chromosomes decreased to approximately 2% at 7 days after reoxygenation. Diplochromosomes were not seen in any of the metaphase spreads at that time. The fraction of metaphase spreads with 92 chromosomes then increased gradually with time and reached 100% at approximately 25 weeks after reoxygenation (Figure 2). Flow cytometric studies confirmed that the cells had become tetraploid; the DNA histograms indicated the presence of only one cell population. The DNA index of this population was 2.19 ± 0.10. Metaphase spreads with 92 chromosomes could not be detected in untreated BEX-c cell populations. Spontaneous tetraploidisation of BEX-c has never occurred since the cell line was established 6 years ago. Moreover, SAX-c metaphase spreads with doubled chromosome number were not seen at any time after reoxygenation.

BEX-c and SAX-c were compared with respect to responses to hypoxia treatment in an attempt to reveal the mechanisms leading to the hypoxia-induced tetraploidisation of BEX-c. Several similarities were detected. The following observations pertain to both lines. The number of cells per dish did not increase during a 24 h hypoxia exposure, i.e. the cells were not able to divide under hypoxic conditions. Approximately 100% of the cells remained attached to the bottom of the glass dishes after 24 h of hypoxia treatment. The fraction of trypan blue-excluding cells decreased gradually with time under hypoxia from approximately 100% for untreated cells to approximately 60% for cells exposed to hypoxia for 24 h. Similarly, plating efficiency decreased gradually from approximately 95% to approximately 25% during a 24 h hypoxia treatment.

Significant differences between BEX-c and SAX-c were also detected. Thus, cell volume increased gradually with time under hypoxic conditions in BEX-c, but remained essentially unchanged in SAX-c (Figure 3). Moreover, some BEX-c cells progressed in the cell cycle and accumulated in G_2 + M during exposure to hypoxia (Figure 4). In contrast, the DNA histogram of SAX-c did not change significantly during a 24 h hypoxia exposure (Figure 4).

The possibility that these differences were related to the expression of p53 and/or pRb was investigated by Western blot analysis (Figure 5). Significant differences between the lines were not detected. Untreated BEX-c and SAX-c cells

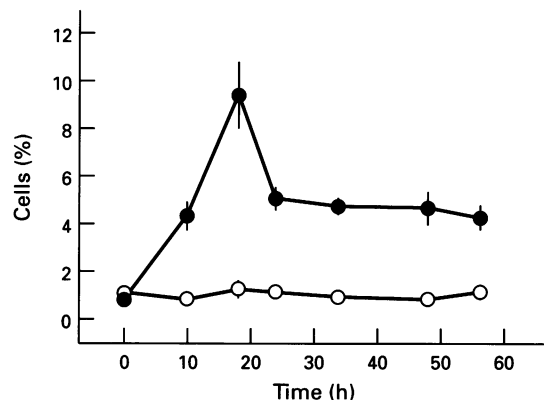

Figure 1 Fraction of BEX-c (●) and SAX-c (○) cells with increased DNA content (larger DNA content than that of stem line G_2 + M cells) *vs* time after reoxygenation. The cells were exposed to hypoxia (O_2 concentration of <100 p.p.m.) at 37°C for 24h. Bars, s.e. of 4–6 experiments. Qualitatively similar results were obtained by using an O_2 concentration of <10 p.p.m. (data not shown).

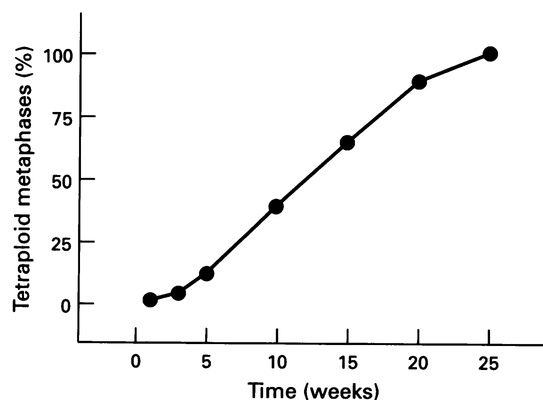

Figure 2 Fraction of BEX-c metaphase spreads with 92 chromosomes *vs* time after reoxygenation. The cells were exposed to hypoxia (O_2 concentration of <100 p.p.m.) at 37°C for 24h. Qualitatively similar results were obtained by using an O_2 concentration of <10 p.p.m. (data not shown).

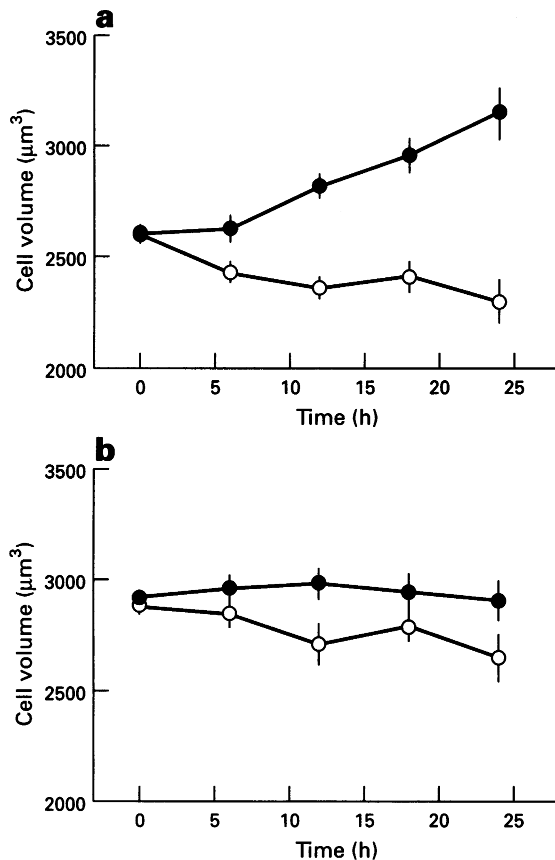

Figure 3 Cell volume *vs* time under hypoxic conditions for BEX-c (**a**) and SAX-c (**b**). The cells were exposed to hypoxia (O_2 concentration of <100 p.p.m.) at 37°C (●). Steel chambers with control cells were flushed with 5% CO_2 in air (○). Bars, s.e. of 4–6 experiments. Qualitatively similar results were obtained by using an O_2 concentration of <10 p.p.m. (data not shown).

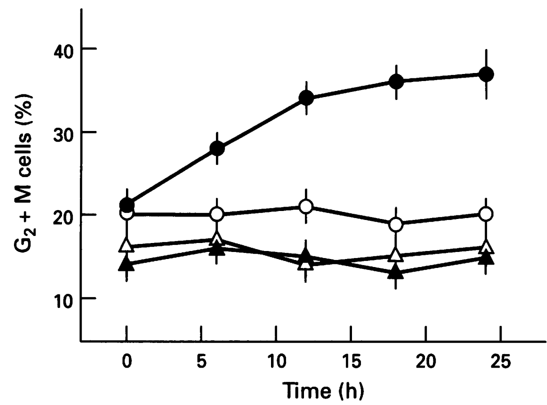

Figure 4 Fraction of cells in G_2 + M *vs* time under hypoxic conditions for BEX-c (○,●) and SAX-c (△,▲). The cells were exposed to hypoxia (O_2 concentration of < 100 p.p.m.) at 37°C (●,▲). Steel chambers with control cells were flushed with 5% CO_2 in air (○,△). Bars, s.e. of 4–6 experiments. Qualitatively similar results were obtained by using an O_2 concentration of < 10 p.p.m. (data not shown).

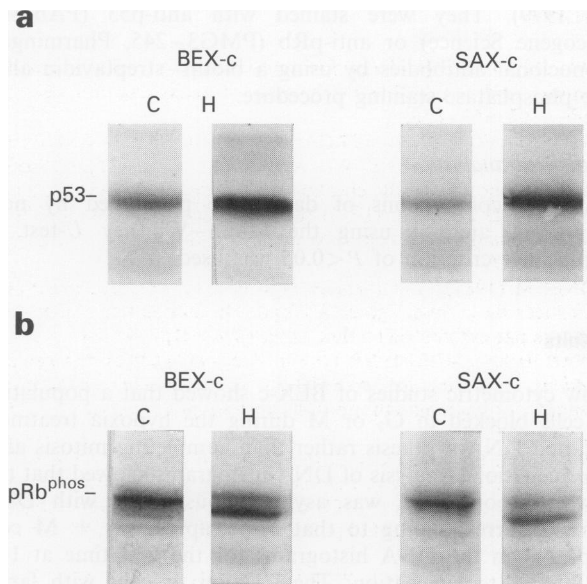

Figure 5 Western blots showing the expression of p53 (**a**) and pRb (**b**) in BEX-c and SAX-c cells. The cells were exposed to hypoxia (O_2 concentration of < 100 p.p.m.) at 37°C for 24 h (H). Steel chambers with control cells were flushed with 5% CO_2 in air (C). The number of cells loaded per lane was 5×10^5. Qualitatively similar results were obtained by using an O_2 concentration of <10 p.p.m. (data not shown).

showed significant expression of both p53 and pRb. Exposure to hypoxia induced increased expression of p53 and hypophosphorylation of pRb.

Discussion

Human tumours in advanced stages of growth are usually hyperdiploid (Merkel and McGuire, 1990). The events leading to hyperdiploidy are not well understood. The most widely accepted model says that the hyperdiploidisation process occurs in distinct steps. Malignant transformation results in genetically unstable diploid tumour cells (Nowell, 1976). Diploid tumour cells frequently increase their DNA content to a DNA index usually within the range of 1.0–1.2 as a consequence of the genetic instability (Nowell, 1976). Such cells might double their chromosome number during an abnormal mitosis or cytokinesis and become tetraploid (Devonec, 1987). Tetraploid tumour cells usually lose non-vital chromosomes gradually, resulting in the development of a stable hyperdiploid stem line (Devonec, 1987).

The studies reported here demonstrate that the tetraploidisation step can be induced by transient hypoxia. Thus, BEX-c was found to develop a new cell subpopulation following exposure to hypoxia and reoxygenation. This subpopulation was verified to be tetraploid by flow cytometric analysis of DNA content and by chromosome counts in metaphase spreads. The oxygen concentration in the hypoxic microenvironment of the cells is probably not critical for the process; the tetraploidisation was induced equally well at 10 and 100 p.p.m. of O_2.

Moreover, the present data are consistent with the hypothesis that tetraploidisation occurs only after diploid tumour cells have acquired excess DNA due to genetic instability (Nowell, 1976; Devonec, 1987). Thus, the DNA index of BEX-c cell populations with a median chromosome number of 46 was 1.10 ± 0.04 (Rofstad *et al.*, 1991).

The mechanisms behind the hypoxia-induced doubling of the number of chromosomes in BEX-c can not be determined unequivocally from the present data. However, some suggestive observations were made. Thus, a significant fraction of the tetraploid metaphase spreads at 18 h after reoxygenation showed diplochromosomes, suggesting that the sister chromatid separation was inhibited by hypoxia. Failure of sister chromatid separation is associated with the lack of synthesis of phosphatase 1 (Doonan and Morris, 1989; Ohkura *et al.*, 1989). The fraction of the tetraploid metaphase spreads at 18 h after reoxygenation that did not show diplochromosomes was also significant. Hypoxia might have caused inhibition of cytokinesis rather than mitosis in these cells.

The permanent tetraploid cell population probably grew out from the tetraploid cells that did not develop diplochromosomes in the first mitosis after reoxygenation.

The tetraploid cells that developed diplochromosomes were in all likelihood not able to exit mitosis and subsequently died. This interpretation is consistent with the initial transient increase in the fraction of tetraploid metaphase spreads and the relative occurrence of tetraploid metaphase spreads with and without diplochromosomes.

BEX-c cells, in contrast to SAX-c cells, increased in volume, progressed in the cell cycle and accumulated in G_2 + M during the hypoxia exposure. It is possible that hypoxia-induced tetraploidisation of tumour cells requires that the cells have capacity to progress in the cell cycle to mitosis or cytokinesis under hypoxic conditions.

The tumour suppressor gene products p53 and pRb are associated with growth arrest following genotoxic stress in an independent manner (Haapajärvi *et al.*, 1995). The difference between BEX-c and SAX-c in growth behaviour under hypoxic conditions can probably not be attributed to p53 or pRb; hypoxia induced increased expression of p53 and hypophosphorylation of pRb in both lines. Recent studies have suggested that transition to tetraploidy following exposure to spindle inhibitors is elevated in p53-deficient cells (Cross *et al.*, 1995; Kaufmann *et al.*, 1995; Powell *et al.*, 1995), but might occur also in p53-intact cells (Cross *et al.*, 1995). BEX-c and SAX-c probably have wild-type p53. Mutations were not detected in any of the cell lines by analysis of exons 5 to 8. Other exons have not been analysed so far. Moreover, it has been shown that hypoxia induces accumulation of p53 protein in cells with wild-type p53, but not in cells which contain only mutant p53 (Graeber *et al.*, 1994).

Regardless of mechanisms, transient exposure to acute hypoxia can induce tetraploidisation of diploid human tumour cells *in vitro*. The possibility thus exists that hyperdiploid tumours might arise *in vivo* as a consequence of transient perfusion.

Acknowledgements
The skilful technical assistance of Berit Mathiesen, Heidi Kongshaug and Kanthi Galappathi is gratefully acknowledged. Financial support was received from The Norwegian Cancer Society.

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