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Growth Factor Requirement, Oncogene Expression and *TP53* Mutations of a Tumorigenic and a Non-tumorigenic Subline of the Human Breast Carcinoma Cell Line, HMT-3909

M.W. Madsen, C. Moyret, C. Theillet and P. Briand

From a human breast carcinoma cell line, HMT-3909, a tumorigenic and a non-tumorigenic subline have previously been described. Cells of both sublines have been characterised as carcinoma cells. In the present work we examined whether differences in growth factor requirements or oncogene expression may explain the difference in tumorigenicity. We found that exogenous growth factor dependence discriminated between the two sublines. No alterations in oncogenes or tumour suppressor genes were demonstrated that could explain the differences in tumorigenicity. The lower growth factor requirement and the higher growth rate of the tumorigenic subline indicates that, in these cells, growth potential may determine the outcome of the tumorigenicity assay.

Key words: growth, oncogenes, human breast cancer, cell line
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INTRODUCTION

ONE OF the prevailing hypothesis of carcinogenesis explains cancer development as a result of alterations in protooncogenes and tumour suppressor genes [1], leading to unregulated expression of growth factors or their receptors and to uncontrolled cell growth. In human breast cancer, the most frequently involved oncogenes and tumour suppressor genes seem to be: *C-ERB-B1* (epidermal growth factor receptor, EGF-R), *C-ERB-B2* (*HER2/neu*), *C-MYC*, *PRAD1/CCND1*, *TP53* and *RB-1*, based on the observation of genetic alterations affecting these genes in primary human breast cancer [2–6]. The unregulated expression of growth factors and their receptors could lead to autonomous growth with a reduced demand for exogenous growth factors. From studies on human breast cancer cell lines in culture, a number of growth factors seems to be involved in "autocrine" (transforming growth factor α (TGF-α); insulin-like growth factor (IGF-I and IGF-II)) or "paracrine" (TGF-β_{1-3}; platelet-derived growth factor (PDGF)) growth regulation [7]. We have previously shown that the immortalisation and decreased growth factor requirement of a non-tumorigenic human breast epithelial cell line were associated with alterations in the expression of *C-MYC*, *C-ERB-B2*, EGF-R and TGF-α [8]. Whether these alterations are important in the pathogenesis of the disease is not known. In model systems, such as human breast epithelial cells in culture, malignant transformation, i.e. acquisition of tumorigenicity in nude mice, has been induced by SV40 Large T antigen [9], and cells bearing both the T-antigen and the *v-ras* oncogene are strongly tumorigenic [10]. Immortalised human breast epithelial cells have been converted to tumorigenic cells by transfection with the *C-ERB-B2* oncogene [11].

In our laboratory, two sublines derived from the same cell line of primary human breast carcinoma cells, have been established in serum-free, chemically defined medium [12]. One subline (HMT-3909S1) is non-tumorigenic, the other one (HMT-3909S8) is tumorigenic. These two sublines were characterised by immunocytochemistry, electron microscopy and cytogenetics and compared with the tissue of origin. The results suggest that the S1 cells are identical to the medullary carcinoma cells and the S8 cells are identical to the invasive ductal carcinoma cells [12]. Since the main difference between the S1 and S8 cells is the difference in tumorigenicity, we studied whether this was reflected in differences in growth rate, growth factor requirements, protooncogene alterations or tumour suppressor gene alterations.

MATERIALS AND METHODS

Cell lines

The two sublines, S1 and S8, were derived from the same parental cell line, HMT-3909, which originated from a primary infiltrating ductal carcinoma with both medullary and intraductal components [12].

HMT-3909S1. Frozen parental HMT-3909 cells in passage 15 were thawed and propagated in H20 medium (see below) as Subline 1 (S1). In passage 18, the cells were tumorigenic in nude mice, but after passage 33 the tumorigenicity was lost.

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HMT-3909*S*8. Frozen parental cells in passage 5 were propagated in H20 medium with 20% fetal calf serum. In passage 33, cells were transferred to the serum-free H21 medium (see below) and propagated in this medium. This subline remained tumorigenic [12].

Growth medium

The chemically defined medium, H20, used for propagation of the S1 cells in monolayer culture was a minor modification of the CDM3 medium [12]. The composition of H20-medium was: DME/F12 (1:1) with the addition of 2.6 ng/ml sodium selenite, 20 ng/ml epidermal growth factor, 0.5 μg/ml (1.38 μM) hydrocortisone, 10 nM triiodothyronine, 100 ng/ml fibronectin (all from Collaborative Research); 2 mM glutamine, 25 μg/ml transferrin, 10 nM dibutyryl cAMP (cyclic AMP), 0.1 mM phosphoethanolamine, 20 μg/ml fetuin, 10 μg/ml ascorbic acid (all from Sigma); trace element mix (1:100) (GIBCO), 3.0 μg/ml insulin (28 IU/mg; Nordisk Insulin Laboratorium, Copenhagen, Denmark), 0.1 nM 17β-oestradiol (Kock-Light Ltd, Suffolk, U.K.), and 0.1 mM ethanolamine (Merck). Usually the media did not contain antibiotics. If added, the antibiotics were penicillin (Leo Pharmaceuticals, Ballerup, Denmark) 250 IU/ml and streptomycin 25 μg/ml. The H21 medium was similar to H20 medium except that 0.01% bovine serum albumin (fraction V) (Sigma), was added.

Growth studies

At Day 0, replicate cultures were seeded at 10^4 cells/cm^2 in a Multidish (Nunclon Delta 24 wells, Nunc, Roskilde, Denmark) coated with Vitrogen-100. On Day 2, growth medium was renewed with experimental medium or with growth medium for those cultures serving as controls. Medium was then renewed 3 times weekly. On Day 8 and, in some experiments also on Day 2, cell numbers were determined by trypsinisation and counting of the cells in Bürker-Türck chambers or in an automatic cell counter (Analys Instrument AS/VDA 140). Final cell number was expressed as the mean cell number of 4 wells. The level of significance was evaluated by the Student *t*-test. For construction of growth curves, cells of 4 wells were counted on each of Days 0, 3, 4, 5, 6 and 7. The doubling time (T_d) was calculated from the growth curve in a semilogarithmic plot.

Southern and northern analysis

Southern and northern blotting were performed as described previously [8]. Purified DNA probes were labelled with [α^{32}P]-dATP (3000 Ci/mmol) (Amersham, Buckinghamshire, U.K.) using Multiprime DNA Labelling kit (Amersham). Prehybridisation and hybridisation of Southern and northern blots were carried out as previously described [8]. The blots were exposed to Kodak XAR-5 or AGFA Structurix-D7pFW films at −80°C. The levels of specific hybridisation signals used in Tables 1 and 2 were scored by eye on a relative basis from + to +++ depending on the intensity of the autoradiographic signal. After hybridisation, the probe was removed and the northern and Southern blots were rehybridised with *GAPDH* (glyceraldehyde-3-phosphate-dehydrogenase) and *β-globin*, respectively, as a control for difference in material loaded on the gel. All Southern and northern experiments were repeated twice on independent DNA and RNA preparations, and the results were reproducible.

Probes

bcl-1: 2, 3 kb Sac I fragment from *bcl-1*; *C-ERB-A1*: 2.0 kb *Eco*RI to *Hind*III fragment from pH-*C-ERB-A1*; EGF-R: 2.4 kb *Cla*I fragment from pE7; *C-ERB-B2*: 0.9 kb *Acc*I to *Eco*RI fragment from pMAC117; *est-1*: 5.4 kb *Eco*RI fragment from pHE5.4; *hst-1*: 0.8 kb *Sac*I to *Eco*RI fragment from pSPT-CS1; *int-2*: 0.9 kb *Sac*I fragment from pSS6; *C-MYC*: 1.2 kb *Pst*I fragment from pRyc 7.4; *c-ras-Ha1*: 2.8 kb *Sac*I fragment from J77; *c-ras-Ki2*: 1.1 kb *Pst*I fragment from pSW11-1 (ATCC no. 41027); *N-ras*: 0.9 kb *Pvu*II fragment from pN-ras; *β-globin*: 1.4 kb *Hind*III to *Eco*RI fragment from Hβ-delta-6; *rat GAPDH*: 1.2 kb *Pst*I fragment from pRGAPDH-13. For references, see [8].

Table 1. *Northern analysis of oncogene expression in HMT*-3909*S*1 *and HMT*-3909*S*8

Oncogene	HMT-3909S1	HMT-3909S8
C-ERB-A1	+	+
C-ERB-B2	+	−
int-2	−	−
hst-1	−	−
C-MYC	++	++
EGF-R	+	+

Northern hybridisation results of protooncogene expression in HMT-3909S1 and HMT-3909S8. The levels of oncogene expression were scored on a relative basis from + to +++ as described in Materials and Methods.

PCR-SSCP analysis (polymerase chain reaction single strand conformation polymorphism)

Genomic DNA were extracted and exons 2, 5, 6, 7, 8 and 9 of the *TP53* gene were analysed by PCR-SSCP and direct DNA sequencing. Primers used to amplify the *TP53* gene were as described by Mazars and associates [5]. SSCP analysis was as described by Spinardi and colleagues [13].

Briefly, sequences corresponding to exons 2, 5, 6, 7, 8 and 9 were amplified in a radiolabelled PCR-reaction and analysed on a non-denaturing polyacrylamide gel. Mutated sequences appear as bands with a variant electrophoretic mobility. These shifted bands were isolated, sequences reamplified by PCR and their nucleotide sequence determined.

RESULTS

Growth curves

The growth rate of the two sublines was evaluated on the basis of consecutive cell counts over a 1-week period (Figure 1). The S8 subline proliferated at a faster rate than the S1 subline as indicated by the doubling times (T_d) of 38 and 55 h, respectively.

Growth factor requirements

The growth factor requirement for growth of the cells *in vitro* was studied in both cell lines by omitting one growth factor or groups of growth factors from the growth medium. Due to the large number of growth factors in the medium, several experiments had to be carried out. In order to be able to pool the results, the final cell numbers of each experiment were expressed relative to a control culture grown in complete growth medium, which was included in each experiment. In the HMT-3909S1 (Figure 2), omission of single factors maximally decreased the final cell number to 65% of the control, and only omission of hydrocortisone resulted in a significantly different growth compared with the control. The most important growth factors in the medium supplement were insulin, transferrin, EGF and

Table 2. Southern analysis of oncogene amplification in HMT-3909S1 and HMT-3909S8

Oncogene	HMT-3909S1	HMT-3909S8	Leucocytes
C-ERB-A1	+	+	+
C-ERB-B2	+	+	+
est-1	+	+	+
int-2	++	++	+
hst-1	++	++	+
bcl-1	++	++	+
C-MYC	+	++	+
c-ras-Ha1	+	+	+
c-ras-Ki2	+	+	+
N-ras	+	+	+
EGF-R	+	+	+

Southern hybridisation results of HMT-3909S1 and HMT-3909S8. The levels of gene amplification were scored from + to +++ in relation to the gene level in normal peripheral leucocytes as described in Materials and Methods.

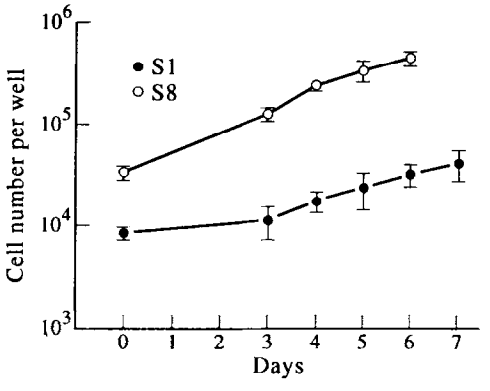
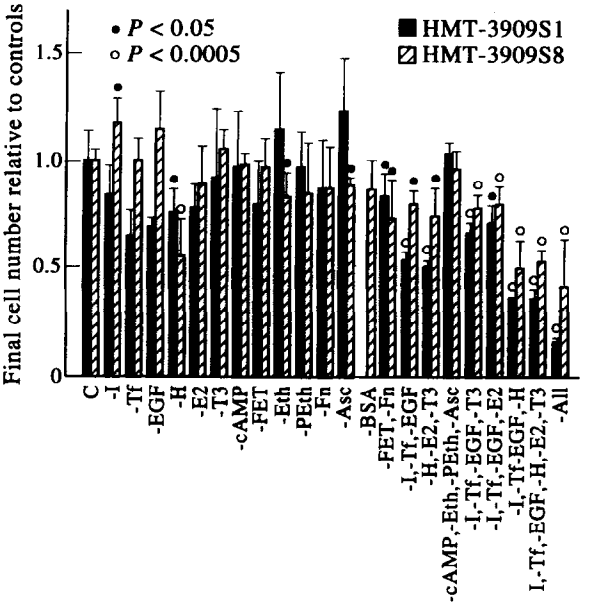


Figure 1. Growth curves of HMT-3909S1 and HMT-3909S8. Points, means; bars, SD.



Factor(s) omitted from medium

Figure 2. Growth factor requirement of HMT-3909S1 and HMT-3909S8. Cells were seeded in 24-well dishes at a density of 1 × 10^4 cells/cm². Two days later (day 0) the medium was renewed in groups of 4 wells with experimental medium lacking one or more of the following substances included in the growth medium: insulin (I), transferrin (Tf), epidermal growth factor (EGF), hydrocortisone (H), oestradiol (E2), triiodothyronine (T3), cyclic AMP (cAMP), fetuin (FET), ethanolamine (Eth), phosphoethanolamine (PEth), fibronectin (Fn), ascorbic acid (Asc) and bovine serum albumin (BSA). At days 3 and 5, experimental medium was renewed. At day 6, the cell number of each well was determined after trypsinisation. Bars, SD.

hydrocortisone; the omission of these four compounds together resulted in a final cell number 35% that of the control value, a difference which was statistically highly significant. Omission of the entire supplement decreased the final cell number to 15% of control values corresponding to the initial cell number, i.e. no growth was observed. In the HMT-3909S8 (Figure 2), omission of hydrocortisone as a single factor resulted in a highly significant decrease in final cell number. Omission of insulin, transferrin, EGF, and hydrocortisone or of all growth factors decreased the final cell number to less than 50% of the control value. Whereas the growth of S1 cells in medium without growth factors was arrested, the final cell number of S8 cells under these conditions was approximately 8-fold the initial cell number, i.e. growth was not arrested, indicating that S1 was more dependent on growth factors than S8. This growth difference between S1 and S8 was statistically significant ($P < 0.001$).

Oncogene overexpression and amplification

By northern blot analysis, the RNA expression levels of a number of genes which have been related to primary breast cancer progression were determined (Table 1), and no significant difference in the level of RNA expression could be observed between subline S1 and S8. Somewhat surprisingly, expression levels of EGF-R, *C-ERB-B2* and *N-myc* mRNAs were slightly higher in subline S1 compared to S8, whereas *C-MYC* expressed significant levels in both cell systems. This study at the RNA expression level was completed by Southern blot analysis for DNA amplification (Table 2), and again no significant differences could be found except for the *C-MYC* gene, whose number of gene copies was slightly increased in subline S8 compared with that of S1. Other tested locations presented identical copy numbers in both sublines.

TP53 *gene mutation*

The presence of mutations that might inactivate its tumour suppressing ability within the coding sequence of the *TP53* gene

was assessed by means of the PCR-SSCP method. A mutation was detected in exon 7, at codon 249 where an A to G transition caused the substitution of Arg by a Gly (Figures 3 and 4). This same mutation was found in both sublines S1 and S8. In order to investigate whether the mutation has occurred during propagation in culture, different passages of both sublines were analysed. Since the mutation could be found as early as in passage 4 of the stem line (Figure 3), we concluded that this *TP53* mutation was probably present in the original breast tumour.

DISCUSSION

In our two sublines of breast carcinoma cells derived from the same tumour, one tumorigenic (HMT-3909S8) and the other non-tumorigenic (HMT-3909S1), we investigated the role of growth rate, growth factor requirement and alterations in protooncogenes and tumour suppressor genes in relation to tumorigenicity. We have shown that growth *in vitro* of the nontumorigenic subline (S1) requires addition of growth factors to the medium, whereas the tumorigenic subline (S8) proliferates—although at a decreased rate—in medium without added growth factors. This correlation raises the interesting question of a possible causal effect of the decreased requirement for exogenous growth factors on the tumorigenic potential.

Indeed, the capability of cancer cells—in contrast to normal cells—to form tumours after transplantation to athymic mice is

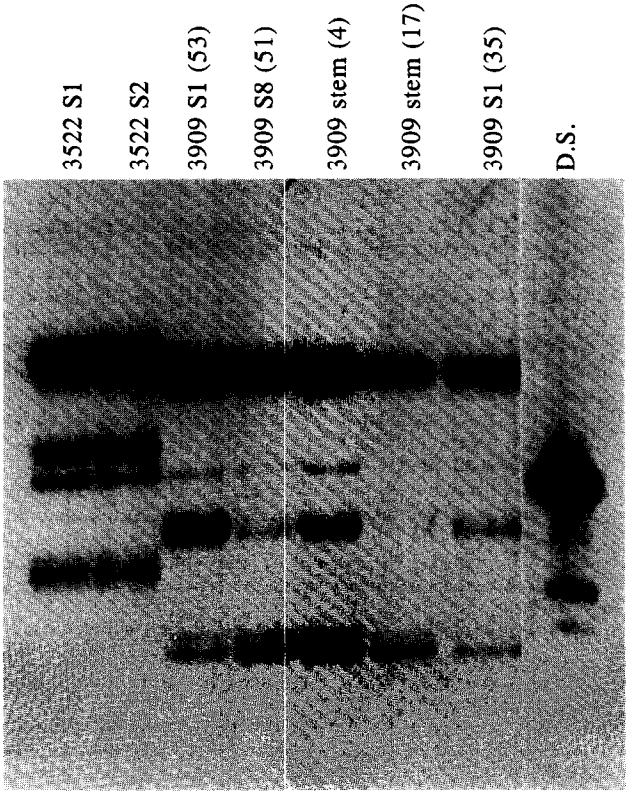


Figure 3. Detection of mutations in exon 7 of *TP53* by SSCP. Different passages of HMT-3909S1 and HMT-3909S8 were analysed by SSCP for detection of mutation in *TP53* as described in Materials and Methods. HMT-3522S1 and HMT-3522S2 were included as controls for wild type *TP53* exon 7. D.S., double stranded DNA. Numbers in parentheses refer to the number of cell passages.

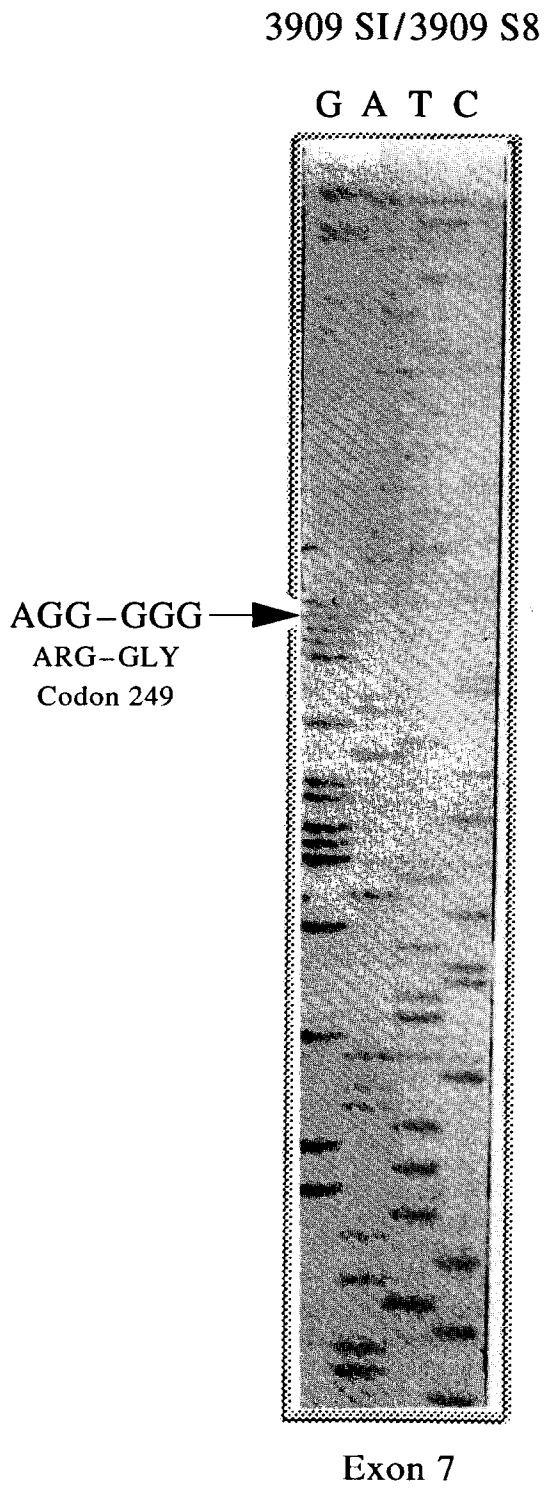


Figure 4. DNA sequence analysis of *TP53* exon 7. Mutated band from the SSCP gel was cut out and reamplified by PCR and sequenced by direct DNA sequencing.

a reflection of a growth advantage. The nature of this growth advantage is not clear. The acquisition of tumorigenicity in transformed fibroblasts in culture is associated with changes in growth properties, i.e. reduced serum requirement, loss of anchorage requirement, loss of contact inhibition, increased saturation density [14]. However, in epithelial systems, none of these growth parameters have correlated consistently with

cellular tumorigenicity in athymic nude mice [14, 15]. More consistently, tumour progression seems related to an increased independence towards exogenous mitogens as was shown in the melanocyte system described by Rodeck and coauthors [16]. Therefore, loss of or decreased requirement for specific growth factors is often found in neoplastically transformed cells in culture, and may lead to a growth advantage [17, 18]. This statement, mainly based on studies of mesenchymal cells, also seems to be valid for human breast epithelial cells as supported by work of Ethier and coauthors [19], who showed a lower growth factor requirement of human breast cancer cells compared with non-malignant breast epithelial cells, demonstrated in serum-free medium.

The growth factor requirements of cancer cells *in vitro* are far from being clarified as exemplified by the different requirements shown by different types of cancer cells. Evaluation of the precise role of single growth factors in promoting cell growth is usually hampered by the presence of poorly defined proteins present in media supplemented with FCS (fetal calf serum) [16]. While the HMT-3909S1 subline has been propagated in chemically defined medium, the S8 subline was selected during a period of growth in serum containing medium and then continued in serum-free medium. By a systematic analysis of individual growth factors as well as groups of growth factors, no single growth factor was revealed that was indispensable for growth. In contrast, a non-tumorigenic, immortalised human breast epithelial cell line, HMT-3522, was totally dependent on EGF and responded to insulin, hydrocortisone and transferrin in earlier passages [20]. These characteristics can be related to those observed for normal human breast epithelial cells in primary culture, which express stringent requirements for insulin, EGF, and cholera toxin. Our results are in accordance with the number of observations relating the shift from EGF dependence to independence to tumour progression [21–24]. A decreased exogenous growth factor requirement is often associated with autocrine secretion of growth factors. Autocrine secretion of TGF-α, which binds to the EGF receptor, has been demonstrated in immortalised breast epithelial cells that had become independent of exogenous EGF [8, 25].

Overexpression of oncogenes and inactivation of tumour suppressor genes has been linked to the cancer phenotype, and many oncogenes code for growth factors or growth factor receptors, whereas many tumour suppressor genes are found to control the cell cycle. We found that a number of oncogenes or genetic loci (*C-MYC* and a series of genes mapping at chromosome 11q13) involved in primary breast cancer were significantly amplified in both cell lines. However, the differences in tumorigenicity between the S1 and the S8 cell lines were not reflected in significant differences in oncogene amplification or expression in spite of differences in growth factor requirement. Moreover, the similarity of S1 and S8 cells was furthermore demonstrated by the presence of a *TP53* mutation in codon 249 of exon 7 in both cell lines. *TP53* mutations have been demonstrated in 40% of primary breast carcinomas [26], although the causal role of *TP53* in breast cancer has only been established in a few cases as in the Li-Fraumeni syndrome [27]. In this study, we have not been able to relate the mutation in the *TP53* gene to the tumorigenic phenotype. However, differences between the two cell lines in other suppressor genes or oncogenes not investigated here cannot be excluded.

The higher *in vitro* growth rate of the S8 subline compared with the S1 subline might indicate that tumorigenicity in nude mice is merely a result of high proliferative activity. However, growth parameters such as doubling time and saturation density were not found to be related to tumorigenicity in a study of tumorigenic and non-tumorigenic clones of MCF-7 cells. All tumorigenic clones required E2 for tumour growth. However, E2 neither affected anchorage independent growth nor growth rate [15]. E2 stimulated production of plasminogen activator, but this parameter was not related to tumorigenicity in accordance with our observations on secreted urokinase-type plasminogen activator in a number of human breast cancer cell lines [28].

In conclusion, exogenous growth factor dependence and growth rate discriminated the tumorigenic from the non-tumorigenic subline of the HMT-3909 human breast cancer cell line. No alterations in oncogenes or tumour suppressor genes were demonstrated that could explain the difference in tumorigenicity between the two sublines S1 and S8. The lower growth factor requirement and the higher growth rate of the tumorigenic subline indicates that, in these cells, growth potential may determine the outcome of the tumorigenicity assay.

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Prophylaxis and Therapy of Mouse Mammary Carcinomas with Doxorubicin and Vincristine Encapsulated in Sterically Stabilised Liposomes

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This study tested the prophylactic efficacies of doxorubicin hydrochloride and vincristine sulphate, encapsulated in sterically stabilised long circulating liposomes, against the spontaneous development of mammary carcinomas in C3H/He mice. Monthly prophylactic intravenous (i.v.) injections of 6 mg/kg doses of liposome-encapsulated doxorubicin (DOX-SL) or 1 mg/kg doses of liposome-encapsulated vincristine (VIN-SL) were begun when retired breeding mice were 26 weeks old. Mice that developed a mammary carcinoma while on the monthly prophylactic protocols were then given weekly i.v. injections of 6 mg/kg DOX-SL or 1 mg/kg VIN-SL to test the therapeutic efficacies of the drugs, and to determine whether the tumours were susceptible or resistant to therapy. The monthly prophylactic injections reduced the incidence of first mammary carcinomas from 87/88 (99%) in untreated mice to 24/42 (57%) in DOX-SL-treated mice and to 26/32 (81%) in VIN-SL-treated mice. Of the mice that developed a mammary tumour while on the prophylactic protocols, 12 of 30 mice were cured by the weekly therapeutic use of DOX-SL, and the growth of 18 tumours was inhibited. The weekly therapeutic use of VIN-SL cured 3 of 8 mice, and inhibited the growth of five tumours. Weekly DOX-SL therapy cured 7 of 22 previously untreated mice. The mean survival of tumour-bearing mice was extended from 24 days in untreated mice to 87 days in DOX-SL-treated mice, which had not received prophylactic treatment. Metastases were found in 29 of 54 untreated mice, and in 3 of 72 mice treated with DOX-SL and VIN-SL. Toxic side effects were limited to a transient weight loss during the weekly treatments. Drug resistance as a result of treatments was not observed.

Key words: liposomes, prophylaxis, mammary carcinoma, metastasis, therapy
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