Further characterization of the defects of skin fibroblasts from cancer patients

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Summary

We have previously shown that skin fibroblasts from breast cancer patients display abnormal growth properties when compared with cells from patients with benign breast lesions. In the present study, we shown that it is possible to define, within the patients previously analysed, a subgroup whose fibroblasts exhibit a significant fraction of cells still synthesizing DNA when growth curves reach a plateau. The phenomenon can also be detected in skin fibroblasts from patients with other types of cancer. In two instances detection of this defect preceded the discovery of the disease.

The high percentage of labelled interphases when cell counts reach a plateau is not due to an increased duration of *S* phase, relative to total cell cycle duration. The data suggest that these cells are delayed in the G_2 phase.

This assay buttresses our previous results, which suggested that at least in some instances cancer is a systemic disease; it could be used for the screening of patients at high risk of cancer.

Research on the patients' somatic cells could shed more light on the process leading to neoplasia, rather than the study of the tumour cells that have already gone through several steps of the evolution to malignancy.

Key words: fibroblasts, skin, cancer.

Introduction

Several studies have shown that apparently normal skin fibroblasts obtained from cancer patients may display behavioural characteristics that are germane to *in vitro* transformation (Azzarone *et al.* 1976, 1980, 1984; Smith *et al.* 1976; Diatloff & Macieira-Coelho, 1979, 1982; Durning *et al.* 1984; Linch *et al.* 1984; Mukherji *et al.* 1984; Schor *et al.* 1985, 1986). This suggests that at least in some instances neoplasia is a systemic disease with repercussions on somatic cells.

Though one of the most common characteristics of transformed cells is to have a high fraction still cycling when the growth curve approaches a plateau (Macieiera-Coelho, 1967*b*), none of the studies mentioned above investigated this particular type of behaviour. This parameter, however, can be rapidly quantified and is useful for the detection of deviations from normalcy (Macieira-Coelho, 1967*b*).

We studied the growth curves and DNA synthesis between seeding and the plateau phase of growth in skin fibroblasts obtained from cancer patients. Results showed that at confluency a significant fraction of cells is still cycling in cultures that originated from some cancer patients as compared to fibroblasts from normal donors.

Materials and methods

Cell cultures

Two embryonic and 10 postnatal skin fibroblast populations were obtained from donors without any known neoplastic process. Fibroblasts were also cultivated from biopsies of uninvolved skin from eight patients with breast cancer and from three types of patients with melanoma, hepatoma and osteosarcoma. The donors had not previously received any chemotherapy, hormone therapy or radiotherapy. Details concerning clinical data on the breast cancer patients and on the 964S, NMS1, BMS2, BMS4, BMS6 and BMS7 cell lines have been described (Azzarone *et al.* 1984). R.D.F was a patient with renal failure and patients A.J., L.B.B. and L.R.B. underwent abdominal surgery for non-neoplastic reasons. Biopsies were obtained from the abdominal or thoracic skin.

Biopsies were put in nutrient medium at 4°C and processed for tissue culture within less than 2 h. Only fragments derived from the superficial skin regions (epidermis and

papillary layer) were used in these experiments. The biopsy was placed in a plastic Petri dish and enough nutrient medium was added, just to nourish the explant without completely covering it. The initial outgrowth around each explant consisted of epithelial-like cells. Then, as is usual with this type of culture, migration and sustained proliferation of fibroblast-like cells occurred. Confluency was reached within 30 days after explantation, then the cultures were transferred by trypsinization (0·25 % in MEM) into plastic bottles. When the cells formed a confluent sheet and no mitoses could be observed, they were subcultivated into new bottles at a 1:2 split ratio. This method was used since it is the one that permits a better approximation of the % of the life-span that has been completed (Macieira-Coelho, 1967*a*). The cells were maintained in Eagle's MEM supplemented with 10% foetal calf serum and 16 μg ml^{-1} gentamycin. Most cultures were used with less than 10 % of their life-span completed. In two instances they were used at different times during their life-span and yielded the same results.

Analysis of growth and DNA synthesis

Confluent cultures were trypsinized, resuspended in complete nutrient medium and seeded into 30 mm plastic Petri dishes. Each day after seeding, duplicate cultures were trypsinized and counted with an electronic counter until no further increase in cell number was observed without renewal of the medium. The counts between identical samples differed by less than 10 % throughout the whole experiment. Tritiated thymidine ([^{3}H]dThd) at a final concentration of 0·1 μCi ml^{-1} (sp. act. 2 μCi mmol^{-1}) was added to duplicated sister cultures each day after subcultivation and 24 h later the cells were prepared for autoradiography (Macieira-Coelho *et al.* 1966). At least 1000 interphases were counted on each slide for the determination of labelled cells. The background was checked by counting the number of grains in control autoradiographs without cells and it was found that a cell with more than five grains could be considered as labelled.

The rate of DNA synthesis and the time of the *S* period were measured according to Stanners & Till (1960). [^{3}H]dThd at a concentration of 0·01 μCi ml^{-1} was added to proliferating cultures. Duplicate samples were removed at hourly intervals thereafter and prepared for autoradiography. The time interval between the first appearance of labelled metaphases and the time when the number of grains over metaphases reaches a plateau during continuous labelling corresponds to the length of the *S* period. In addition, the method also gives information on the time course of DNA synthesis. For the determination of the number of grains over metaphases, the grain counts performed on 100 labelled mataphases at each hour after adding the precursor, were plotted as histograms. The cumulative percentage of such labelled metaphases plotted against grain counts on probability paper gives a straight line. The intersection of the straight lines with the 50 % line on the paper, represents the peak values of metaphase grain counts. A hundred metaphases were scored to determine the % of labelled metaphases.

Results

Fig. 1 shows typical growth and DNA synthesis curves of normal human fibroblasts. After an initial lag phase, the three cultures entered the logarithmic phase of growth, which was characterized by a high percentage of cells synthesizing DNA. However, when cultures reached the stationary phase of growth, i.e. when confluency was observed and no further increase in cell counts could be detected, the percentage of cells synthesizing DNA fell immediately to very low levels (less than 5 %). This behaviour is common to the three

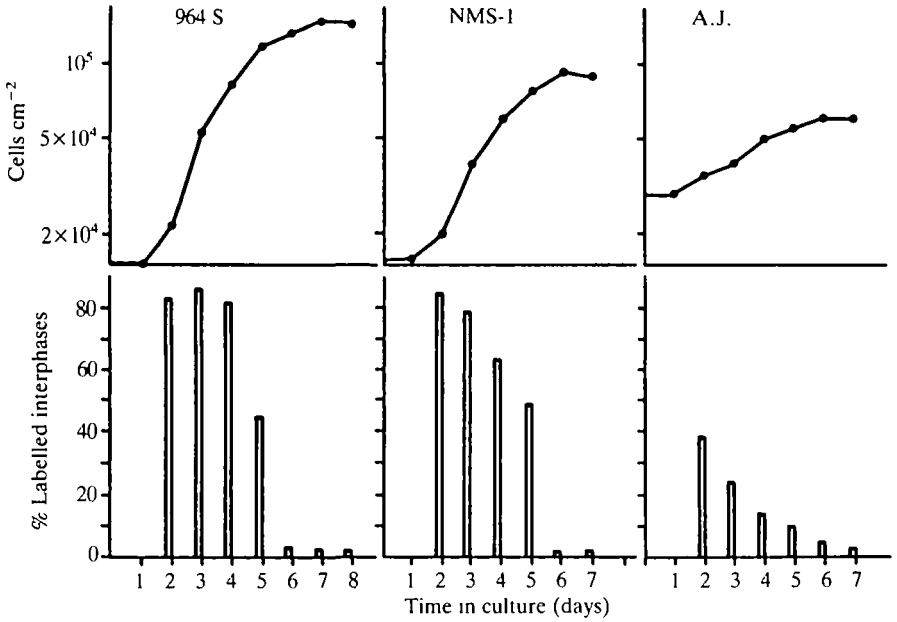

Fig. 1. Growth curves, plotted semilogarithmically, of embryonic (964S) and adult (NMS-1 and A.J.) normal skin fibroblasts. The columns indicate the % labelled cells every 24 h after subcultivation.

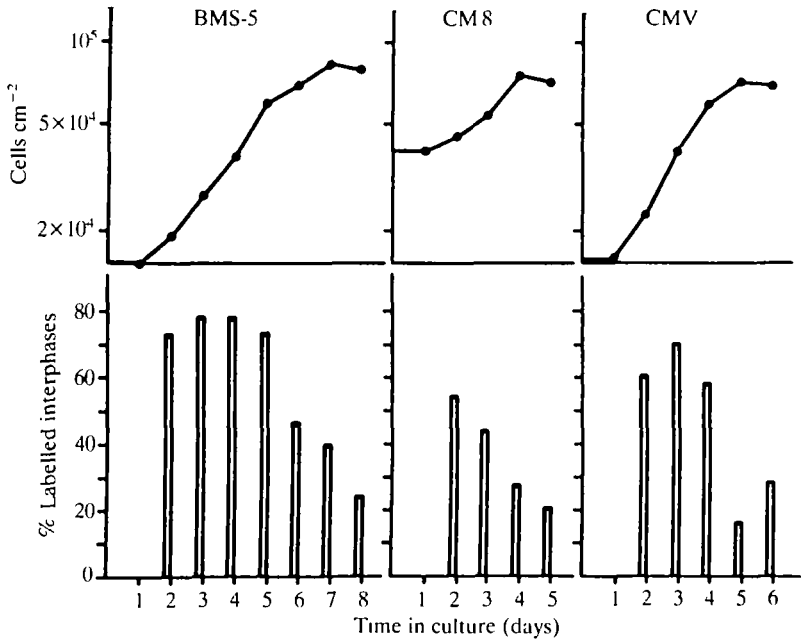

Fig. 2. Growth curves plotted semilogarithmically, of skin fibroblasts from patients with breast cancer (BMS-5, CM8 and CMV). The columns indicate the % labelled cells every 24 h after subcultivation.

cell lines regardless of the age of the donor and the growth rates before the stationary phase, which are different for each cell line. These experiments were performed on the 14th, 8th and 12th passages, respectively. Twelve different skin fibroblastic cultures obtained from non-neoplastic donors of different ages and sex behaved similarly.

However, in skin fibroblast cultures derived from three breast cancer patients (Fig. 2) a significant number of labelled interphases could still be detected (22 %, 28 % and 25 %, respectively) after the plateau in the growth curves. These experiments were performed on the 7th, 8th and 8th passage, respectively.

It is noteworthy that the BMS5 cell line was obtained from a patient operated on for a benign breast lesion, whose mother had developed a breast cancer (Azzarone *et al.* 1984). Periodical testing of the patient for 3 years after the first surgery permitted the early detection of breast cancer (Azzarone *et al.* 1984). A similar alteration was also found in the CMV cell line, which was derived from the abdominal skin of a patient with breast cancer. This shows that abnormal *in vitro* behaviour is not limited to fibroblasts derived from tissues surrounding the primary lesion (Azzarone *et al.* 1976, 1984; Durning *et al.* 1984).

Persistence of a high percentage of labelled interphases was also found, with a similar incidence, in confluent cultures obtained from patients affected with melanoma, hepatoma and osteosarcoma. Fig. 3 shows experiments on single cell lines that are representative of cancer patients with different types of tumours. The melanoma and the osteosarcoma fibroblasts were tested early (17th and 14th passage) and late (40th and 38th passage) during their life-span and yielded identical results.

It is interesting that, in the experiment concerning the patient bearing a hepatoma, the presence of the neoplasia was ascertained only post mortem. It is also interesting that the fibroblasts from the osteosarcoma patient grew slowly; indeed it took 9 days for the growth curve to reach a plateau. However, when the plateau was reached the fraction of cells synthesizing DNA was still 20 %. We never observed a fall in cell counts after the plateau was reached.

Table 1 presents details of the growth characteristics observed in skin fibroblastic cultures obtained from normal donors and from patients affected with different types of neoplasia. The 12 control cell lines exhibited less than 7 % of labelled interphases on the second day of the plateau phase of the growth curves; this behaviour is independent of the age of the donor, the initial seeding density, the plateau density and the time required to reach the plateau. On the contrary, in cultures from different cancer patients at least 20 % of the interphases were still labelled on the second day of the plateau phase. Even in these cell lines this behaviour does not depend on the donor's age, seeding density, plateau density or time required to reach the plateau. Moreover, each line derived from cancer patients exhibits values for seeding density, plateau density or time necessary to reach plateau similar to

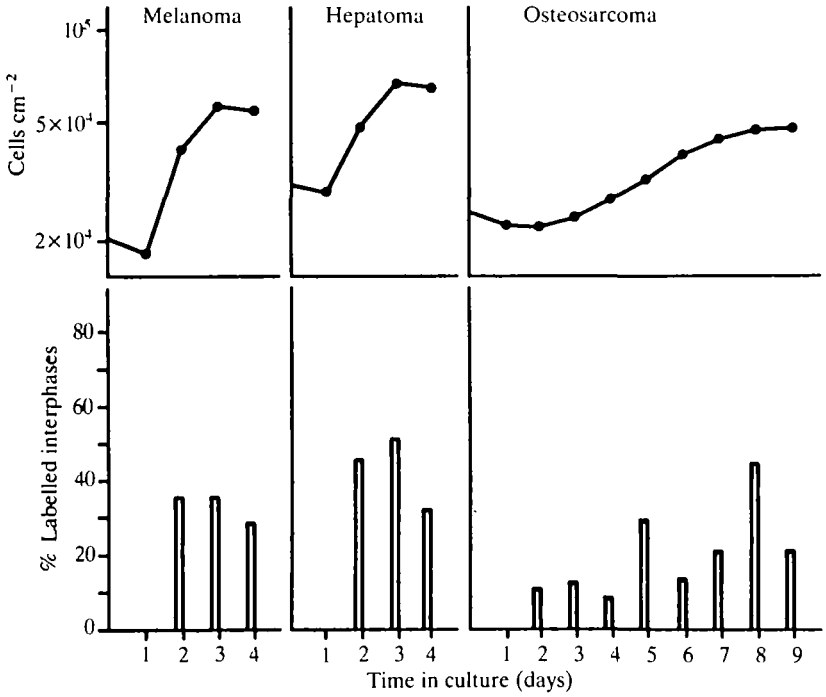

Fig. 3. Growth curves plotted semilogarithmically, of skin fibroblasts derived from different cancer patients (melanoma, hepatoma and osteosarcoma). The columns indicate the % labelled cells every 24 h after subcultivation.

Table 1. *Growth characteristics observed in fibroblastic cultures*

Cell line	Donor's age/sex	Initial seeding (cells cm^{-2})/	Final density (cells cm^{-2})/ time (days) required to reach plateau	% Labelled cells on the 2nd day of plateau phase
Controls				
964S	Embryo/M	12 000	130 000/7	2 (±1)
ICIG IX	Embryo/M	15 000	118 000/6	1·7 (±0·6)
BMS2	41/F	20 000	80 000/7	7 (±1·3)
BMS4	40/F	15 000	90 000/6	7 (±1·1)
BMS6	29/F	15 000	35 000/9	3 (±0·9)
BMS7	45/F	20 000	55 000/6	3 (±0·6)
NMS1	24/F	12 000	90 000/6	1·6 (±1)
A.J.	24/F	13 000	45 000/6	1·5 (±0·7)
L.R.B.	50/M	12 000	80 000/7	3 (±1·2)
L.B.B.	50/F	10 000	40 000/9	4 (±0·9)
R.D.F.	40/F	15 000	60 000/4	3·3 (±1·3)
N.S.	30/M	11 000	53 000/7	7 (±1·3)
Cancer patients				
BMS5	30/F	13 000	80 000/7	22 (±3·3)
CM8	58/F	40 000	77 000/4	25 (±3·7)
CMV	35/F	12 000	78 000/5	28 (±4·2)
Melanoma	30/M	20 000	60 000/3	30 (±4·5)
Hepatoma	61/M	35 000	70 000/3	30 (±4·5)
Osteosarcoma	20/F	25 000	48 000/8	28 (±3)

those observed in one of the control cultures; nevertheless they display abnormal labelling indices on the second day of the plateau phase.

We further investigated the possible role of the culture conditions by varying the initial seeding density, in different cell lines derived from both control and neoplastic patients, and we analysed the effects on the final density/time required to achieve the plateau and percentage of labelled cells at the plateau phase. Fig. 4 shows that in embryonic cells the initial inoculum does not influence the plateau density. The cultures seeded at the higher inoculum reach, within 3

days, the same final density obtained after 7 days in the cultures seeded at the lower inoculum. In both instances when the cultures reached the plateau density, independently of the time taken, the percentage of cells synthesizing DNA fell to very low levels. This confirms our previous results obtained with embryonic lung fibroblasts early in their life-span (Macieira-Coelho & Azzarone, 1982). On the contrary, in skin fibroblasts from adult donors derived from both control and neoplastic patients the initial seeding density influences the final density: cultures seeded at the higher inoculum always reach a higher plateau density. We found similar behaviour in embryonic lung fibroblasts that had exhausted more than 50% of their life-span (Macieira-Coelho & Azzarone, 1982). We interpreted this phenomenon as representing the decrease in growth potential that characterizes aging of normal cells *in vitro*. Indeed the decline in the saturation density accompanies the exhaustion of the division potential. Plating at lower inocula causes a higher number of doublings and accelerates the decline in saturation densities.

Since it has been shown, for several parameters, that adult fibroblasts behave *in vitro* like embryonic aged cultures (for review, see Macieira-Coelho, 1983), it is not surprising to observe this behaviour in adult fibroblastic cultures, early in their life-span. It is interesting that varying the plateau density within the same cell line does not affect the final percentage of labelled interphases. Indeed, in cultures from controls the percentage of labelled cells on the second day of the plateau phase falls to very low levels independently of the final density (Fig. 4), whereas in cultures from neoplastic patients this percentage stays elevated independently of the density achieved at the plateau phase (Fig. 4). This shows that this abnormal behaviour does not depend on the final density achieved or on the number of days necessary to reach the plateau.

To see if the high labelling indices could be due to a prolongation of the duration of S period, the latter was measured in skin fibroblasts obtained from the osteosarcoma patient (Fig. 5) and from a control (Fig. 6). As shown in Figs 5 and 6 the first labelled metaphases were observed during the fifth hour of labelling and the peak metaphase grain count was saturated by the 10th h. This corresponds to an S period of 6 h, identical to that previously reported for normal human fibroblasts (Macieira-Coelho *et al.* 1966). The rate of DNA synthesis was also the same as that previously found. The cell line from the osteosarcoma patient was chosen for this type of experiment since it displayed the longest delayed resting phase that we observed. Our data show that even in this situation the duration of S phase did not vary.

Since high indices of labelled cells at the plateau phase were not due to an increased duration of the S period, we tried to understand whether this behaviour could be attributed to the presence of cells delayed in the cell cycle. This was done by measuring the time spent in G_2; the percentage of labelled metaphases from the same experiment was plotted against time (h). As can be seen in Fig. 7 100% of the metaphases were labelled from the fifth hour on in the control cultures. On the other hand, in the cultures obtained from the osteosarcoma patient 100% labelled metaphases were not reached within the experimental time. If 13 h after adding the labelled precursor there are

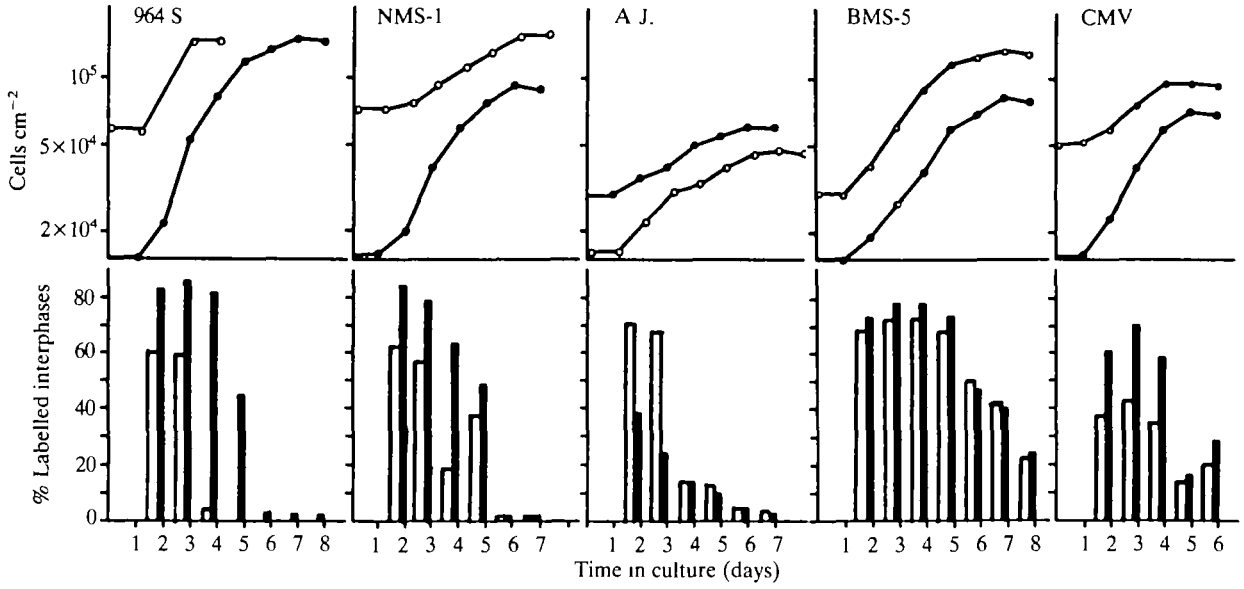

Fig. 4. Growth curves plotted semilogarithmically, of skin fibroblasts from normal donors (964S, NMS-1 and A.J.) and from cancer patients (BMS-5 and CMV) initially seeded at different inocula. The columns indicate the % labelled cells every 24 h after subcultivation.

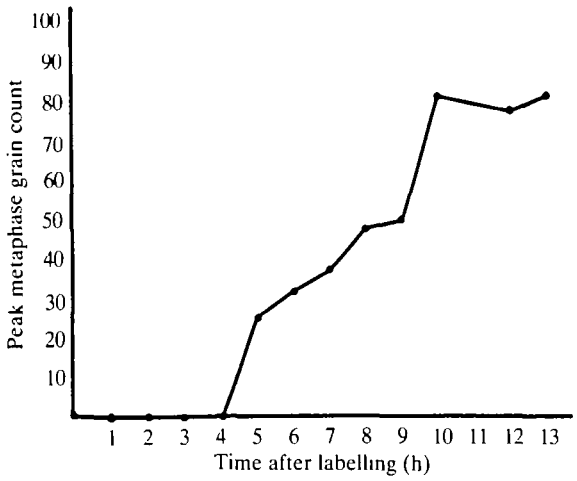

Fig. 5. Peak metaphase grain count determined during continuous labelling of skin fibroblasts obtained from patients with an osteosarcoma.

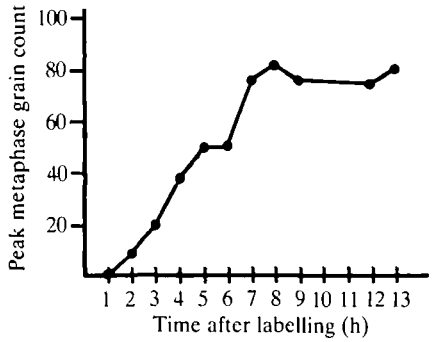

Fig. 6. Peak metaphase grain count determined during continuous labelling of skin fibroblasts obtained from a normal donor.

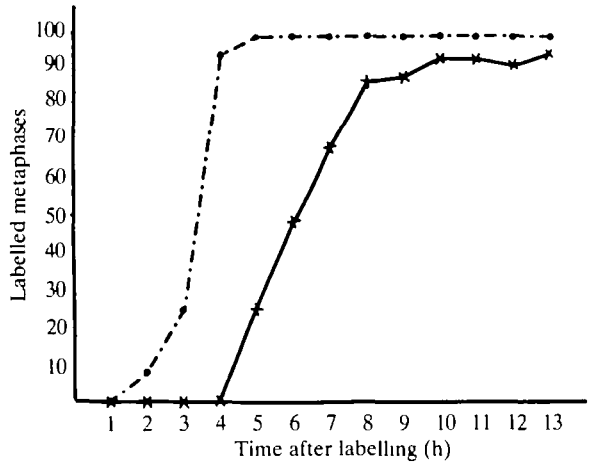

Fig. 7. Percentage labelled metaphases determined during continuous labelling of skin fibroblasts obtained from a normal donor (–·–·–·) and from the patient with an osteosarcoma (×——×).

still non-labelled cells, this means that these cells spent at least this much time in G_2. This shows that in cultures from the neoplastic patient there is a fraction of cells delayed in the cycle. Since the saturation densities do not vary in the following days and since there is no detectable presence of cells floating in the medium, we suggest that the stability of the saturation densities is not due to a continuous loss and renewal of cells.

Discussion

Our results show that skin fibroblasts from some patients with different types of cancer may exhibit, in contrast to embryonic and adult cells, a high percentage of labelled interphases when the growth curves reach a plateau. This deviation from normalcy is commonly found in transformed fibroblasts (Macieira-Coelho, 1967*b*). These results confirm previous findings showing that deviations from normalcy of the somatic cells can be found in patients with different types of tumours (Azzarone *et al.* 1976, 1984; Smith *et al.* 1976; Chauduri *et al.* 1975; Durning *et al.* 1984; Mukherji *et al.* 1984; Schor *et al.* 1985). The defect does not depend on the age of the donor, the site of biopsy, the age of the culture, the initial inoculum, the plateau density or the time required to achieve the plateau. This agrees with previous data that suggested that abnormal types of behaviour of fibroblasts from cancer patients were not affected by these parameters (Smith *et al.* 1976; Azzarone *et al.* 1984; Durning *et al.* 1984; Schor *et al.* 1985). The defect is not due to an increased duration of the S phase, relative to total cell cycle duration. Analysis of the percentage of labelled metaphases together with the stability of the final cell densities suggest that these cells are delayed in G_2 phase. This indicates that a fraction of the population is not competent to perform a normal cell cycle and creates a bottleneck before division. The percentage of labelled cells depends on the fraction that took up the precursor and on the division of these cells and the unlabelled ones. The creation of a bottleneck could alter the balance between these two compartments with a relative predominance of the labelled one.

We have recently shown that skin fibroblasts from breast cancer patients display the ability of increasing progressively their terminal cell densities during serial subcultures at 1:1 split ratio and that this behaviour is probably due to the presence of cell subsets less sensitive to contact inhibition of cell division (Azzarone & Macieira-Coelho, 1982; Azzarone *et al.* 1984). These features demonstrate an increased heterogeneity in these cell populations, detectable by their relative sensitivity to contact inhibition of growth, which could contribute to the imbalance

between the different cell compartments. The increase in labelled cells does not seem to be due to DNA repair since the grain count has a normal distribution with a mode number of 80 grains per cell. The percentage of cells with a low number of grains, which could represent the fraction of cells undergoing DNA repair, was less than 2%. On the other hand, the defect in the cell cycle progression described above could be germane to previous observations dealing with the higher sensitivity to radiation in the G_2 period of fibroblasts from high risk cancer patients (Parshad *et al.* 1985). This could cause a further delay in the progression in labelled cells. From these data, together with our previous observations on the response to ionizing radiations of skin fibroblasts from some patients at high genetic risk of cancer or with cancer (Diatloff & Macieira-Coelho, 1979, 1982; Azzarone *et al.* 1980), one might wonder if the patients presenting this defect in cell cycle progression in their somatic cells could constitute a group of patients more sensitive to environmental oncogenic factors and/or to radiotherapy or chemotherapy, and therefore more prone to develop a primary or a secondary neoplasia.

This type of analysis may constitute an easy and sensitive assay for the screening and early detection of patients at very high risk of cancer; indeed in two instances the detection of this abnormal behaviour preceded the discovery of the disease. It has been suggested that skin fibroblasts from patients at high genetic risk of cancer or bearing cancer, behave at least in part as embryonic cells (Pfeffer *et al.* 1976; Schor *et al.* 1985). On the other hand, embryonic cells, in contrast to adult ones, exhibit *in vitro* the production of autocrine growth factors (Clemmons, 1983); thus the increased labelling indices in skin fibroblasts from some cancer patients could be due to the acquisition of similar autocrine properties. Alternatively, an altered homeostasis and/or expression of some cellular genes, recently implicated in the control of cell cycle progression (Pardee *et al.* 1985) could be linked to the acquisition of this abnormal property.

We suggest that research on the modified somatic cells could shed more light on the process leading to neoplasia rather than the study of tumour cells that have inevitably already undergone profound changes. Indeed the presence of fibroblast populations with abnormal properties could represent an anarchistic milieu (Smith *et al.* 1987), which may influence the subsequent development of an epithelial tumour by virtue of a persistent dysfunction in normal epithelial mesenchymal interactions (Schor *et al.* 1985). In any case the modifications described to date in the somatic cells of cancer patients indicate complex alterations whose mechanisms are for the moment far from being elucidated.

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