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Cell cycle kinetics, tissue transglutaminase and programmed cell death (apoptosis)

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Studies were undertaken on a highly metastatic hamster fibrosarcoma cell line with a view to assessing whether cells entering into apoptosis, measured by counting the number of transglutaminase mediated detergent insoluble envelopes, has any synchrony with a particular phase of the cell cycle. A double exposure of thymidine was used to block cells in early S-phase. Flow cytometry in combination with [^{3}H]thymidine incorporation into DNA was used to assess the degree of synchrony and progression through the different phases of cell cycle. The apoptotic index was found to be at its maximum in mid-S-phase. Measurement of transglutaminase activity in each phase of the cell cycle indicated that the specific activity was also at its greatest during mid S-phase. The level of enzyme was relatively unchanged throughout the cell cycle indicating that the regulation of transglutaminase activity occurs primarily through effects on catalytic activity rather than enzyme synthesis.

Cell cycle; Tissue transglutaminase; Apoptosis

1. INTRODUCTION

Programmed cell death, or apoptosis, is a process whereby cells die in response to a specific physiological signal. Apoptosis was first described in 1972 [1] and is now known to play a role in a number of biological processes including embryogenesis tissue remodeling and tumour regression [2]. Morphologically the process is characterised by cells undergoing both nuclear and cytoplasmic condensation resulting in the formation of apoptotic bodies which are normally rapidly phagocytosed by neighbouring cells or by macrophages. Chromatin condensation is thought to result from activation of an endogenous Ca^{2+} and Mg^{2+} dependent endonuclease [3], although in some systems this process is not always apparent [4]. Apoptotic body formation is thought to require increased expression of tissue transglutaminase [5,6]. Both those enzymes carry out irreversible reactions on cellular molecules. Transglutaminases (EC 2.3.2.13) catalyse a Ca^{2+}-dependent acyl transfer reaction between the γ-carboxamide group of a peptide bound glutamine residue and the ε-amine group of peptide-bound lysine resulting in the production of highly crosslinked protein aggregates [7]. Three forms of transglutaminase each with its own distinct gene have been well characterized: the plasma factor XIII, the keratinocyte enzyme, and the tissue transglutaminase [8,9]. While the physiological role of the first two enzymes is well understood, the role of the tissue enzyme is still unknown. Recent studies suggest the cytosolic form of the tissue enzyme is involved in the physiological cell death programme where increased expression of the enzyme leads to the formation of a highly crosslinked detergent insoluble protein shell [10]. Such a role is comparable to that of the keratinocyte transglutaminase where activation of this enzyme leads to the assembly of the insoluble cornified envelope beneath the plasma membrane of the terminally differentiating keratinocyte [11]. Transglutaminase mediated apoptotic body formation provides a potential method for measurement of cells undergoing apoptosis in particular in a cell population where the apoptotic index is very low. For example, in malignant cells there is now increasing evidence to suggest that aberrant apoptosis leading to an imbalance between cell proliferation and cell death may be an important feature in the development of the metastatic phenotype [10,12]. Studies with a malignant hamster fibrosarcoma cell line have indicated a direct correlation between transglutaminase activity and the spontaneous levels of apoptosis occurring in these cells [10], and an indirect correlation between transglutaminase activity and metastatic potential [10,13,14]. Of importance in this scenario is whether apoptosis can take place at any point in the cell cycle or whether it is necessary for cells to leave the cell cycle and become quiescent before the apoptotic programme can begin. This fundamental question is important in the overall understanding of apoptosis itself and in the

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development of new anticancer therapies designed to induce apoptosis.

In order to answer this question we have measured apoptotic body formation and transglutaminase activity in the different phases of the cell cycle of a highly metastatic hamster fibrosarcoma cell line.

2. EXPERIMENTAL

A highly metastatic hamster fibrosarcoma cell line (Met B) was grown in supplemented DMEM (Dulbecco's modified essential medium) as previously described [10]. Cell numbers were monitored by direct counting using a haematocytometer.

Exponentially growing cells (approximately 1.5×10^5/ml) were treated for 16 h with 2 mM thymidine. Cells were then washed, resuspended in a drug-free medium for 8 h and subsequently treated with the same amount of thymidine for an additional 16 h. In another experiment, exponentially growing cells were treated with a single dose of 4 mM thymidine for 16 h. The cytotoxic effect of thymidine was monitored by Trypan blue exclusion method.

The degree of synchrony was assessed at various times following the release of the thymidine blockage by two different methods.

2.1. *Incorporation of [^{3}H]thymidine (33 mCi/mmol) into DNA*

Cells were incubated at 37°C with 2 μCi of radiolabelled thymidine for 30 min. Cells were washed with 1 ml of 5% (w/v) TCA to remove the acid-soluble fraction. The precipitate was washed twice with 5% TCA and solubilised in 0.5 ml of 1 M KOH. 0.2 ml portions of the alkaline extract were measured for radioactivity in liquid scintillation cocktail (Packard Instruments) using a Packard A300 CD Spectrophotometer.

2.2. *Flow cytometry*

Cells (approximately 1×10^6) were harvested at various times following the release of thymidine, then washed with PBS and stained with nucleic acid intercalating dye, propidium iodide (PI), using a Cycle Test staining Kit (Becton Dickinson). Briefly, cells were incubated with trypsin, in stabilising buffer; the resulting nuclei were then stained with PI following treatment with trypsin inhibitor and RNase. Flow cytometric analysis was carried out on an 'Orthocyte bench top cytometer' equipped with a mercury/xenon arc lamp. At least 10^4 nuclei were measured using a standardised (stored) analysis protocol, with a slow flow rate of 5 μl/min. Histograms were stored and files reanalysed using the MULTICYCLE DNA analysis package. Samples representing a G0/G1 peak with coefficient of variation (*cv*) greater than 10, were excluded from this study.

Transglutaminase activity was measured in cell homogenates by the Ca^{2+}-dependent incorporation of [^{14}C]putrescine into *N,N*-dimethylcasein [11]. Activity is expressed as units (1 unit being equivalent to 1 nmol of putrescine incorporated per h under the conditions of the assay). Protein concentration was determined by Lowry method [16]. Transglutaminase antigen was measured by a quantitative sandwich ELISA [17].

For assessment of the apoptotic index, detergent-insoluble apoptotic envelopes were isolated from synchronized cells [12]. Apoptotic index is expressed as the number of apoptotic envelopes obtained from 10^6 cells.

3. RESULTS AND DISCUSSION

Both aphidicolin (DNA polymerase α inhibitor) and thymidine (thymidine kinase inhibitor) were used initially in order to establish which drug would produce satisfactory synchrony in the hamster fibrosarcoma (Met B). The most efficient cell synchrony was obtained

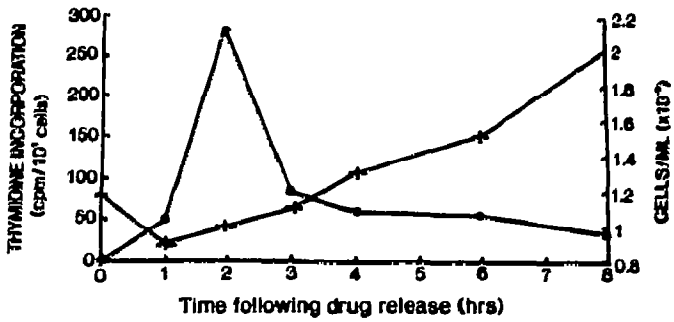

Fig. 1. DNA synthesis and cell replication in thymidine synchronised cells. Cells were synchronised with 2 mM thymidine as described under experimentals. (●) DNA synthesis was assessed by following the incorporation of [^{3}H]thymidine into TCA precipitable components. (+) Cell numbers were determined by direct microscopic counting using a haemocytometer.

by exposing cells to a double block with 2 mM thymidine. During the first exposure to thymidine, cells engaged in DNA synthesis are blocked in S-phase, while cells in other phases continue growth and accumulate at G1/early S-phase. On removal of the drug, the whole population passes through the cell cycle into G1-phase, which takes about 6–8 h. Further exposure of cells to thymidine at this stage for 14–16 h results in blockage of the cell population at early S-phase. This protocol resulted in synchronous population of Met B cells which produced satisfactory synchrony (see below). Using [^{3}H]thymidine incorporated into DNA as a marker of DNA synthesis, Fig. 1 shows that following imposition of the drug (0 h) complete inhibition of DNA synthesis is obtained. However, on removal of the drug cells move through the cell cycle and DNA synthesis rapidly resumes reaching a maximum by 2–3 h, then decreases to minimal level between 4 and 6 h as the cells undergo mitosis and divide as indicated by the increase in cell number. Cell synchrony was also assessed at various times following exposure to thymidine by flow cytometry. DNA histograms (Fig. 2) indicate that exponentially growing cells are blocked in early S-phase and generally constitute greater than 85% of the cell population. By 6 h following the removal of drug, cells have passed through G2/M and into G0/G1 phase by 8 h. This result confirms the data shown in Fig. 1 and shows that this highly metastatic hamster fibrosarcoma cell line takes a period of approximately 8 h to progress through cell cycle before synchrony is lost.

Measurement of transglutaminase activity (U/mg protein) in the different phase of cell cycle following removal of thymidine indicated little change in activity up to 6 h (G2/M-phase). After this time, a slight but significant increase was detected as the cells progressed from G2/M to G0/G1 and finally to loss of synchrony (Fig. 3A). In contrast, when transglutaminase activity is expressed per ng of enzyme protein, the specific activity was found to show a significant increase after 2–3 h

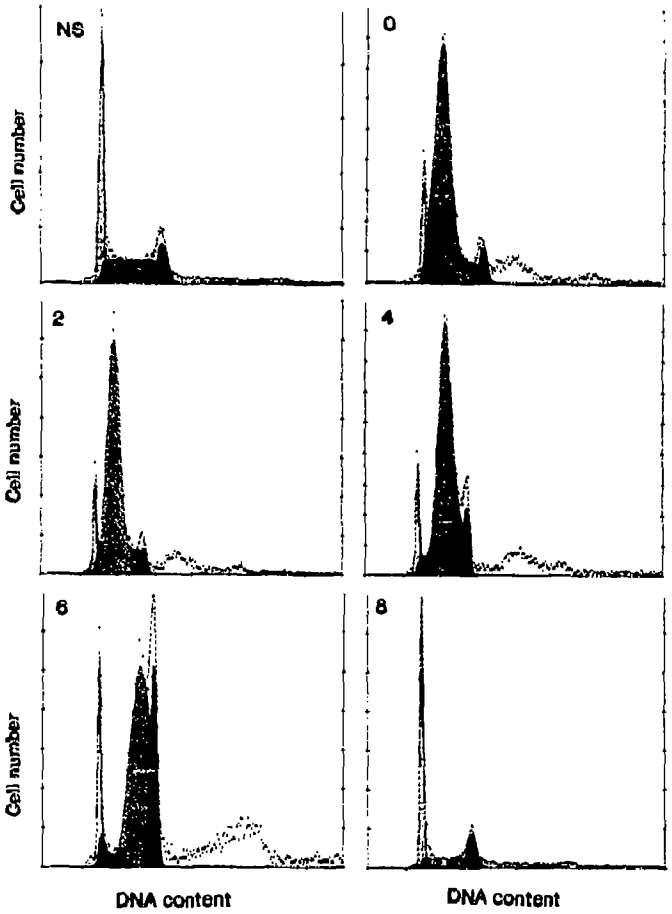

Fig. 2. DNA histograms from thymidine-synchronised Met B cells. At the time indicated in each figure, cells were released from thymidine blockade, stained with propidium iodide and analysed by flow cytometry as described under experimental. NS, non-synchronised cells; dotted, G0/G1-phase; hatched, S-phase; and black, G2/M-phase.

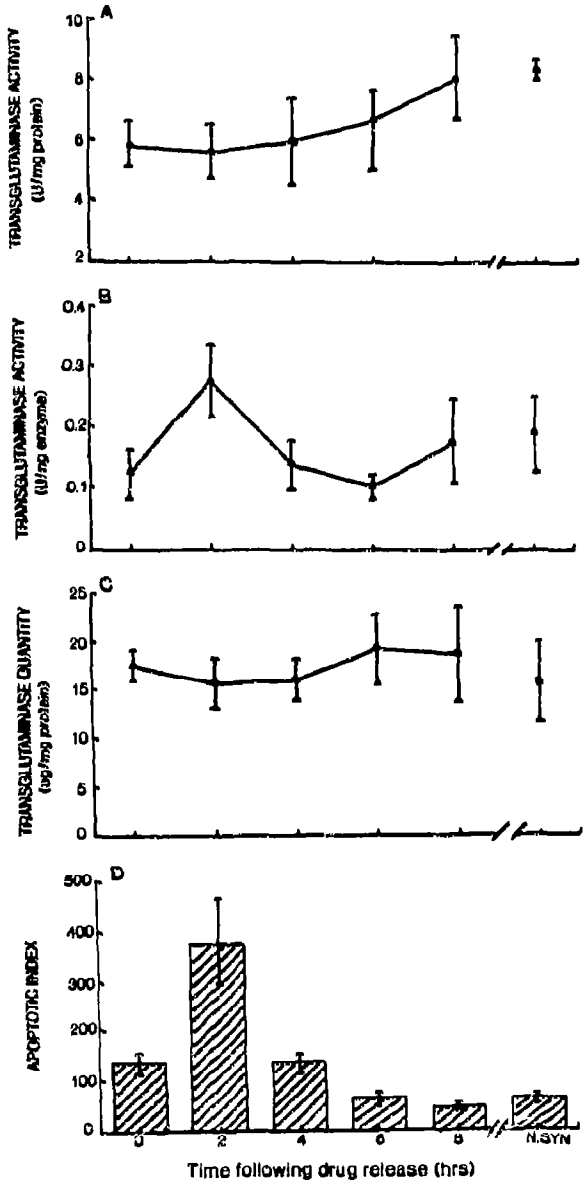

Fig. 3. Transglutaminase activity (U/mg protein (A), U/ng enzyme (B)), transglutaminase level (C) and apoptotic index (D) in synchronised Met B cells compared to non-synchronised cells (N.SYN). The results represent the average data from 5 experiments.

corresponding to mid S-phase of the cell cycle (Fig. 3B). Measurement of enzyme antigen (Fig. 3C) indicated this increase was not due to altered expression of the enzyme but more likely due to a direct effect on the catalytic activity of the enzyme itself.

Measurement of apoptosis in the synchronised Met B cell line was undertaken by counting the number of transglutaminase-mediated detergent-insoluble apoptotic envelopes resulting from a fixed number of cells. However, before using this technique, it was necessary to demonstrate that in this hamster fibrosarcoma, isolated detergent insoluble envelopes are indeed derived from cells undergoing morphological changes typical of apoptosis. Non-synchronised Met B cells were therefore first separated into enriched apoptotic and non-apoptotic population using fractionation on a Percoll step-density gradient [18]. Examination of these two populations by phase microscopy indicated the dense apoptotic population to contain a high percentage of cells with obvious surface blebbing typical of apoptosis (Fig. 4). Isolation of detergent insoluble envelopes from the enriched apoptotic and the non-apoptotic populations demonstrated that greater than 90% of the envelopes were present in the enriched apoptotic cell population. Measurement of the number of apoptotic envelopes in the synchronised Met B cells indicated that by far the greatest level of apoptosis was found in mid S-phase although spontaneous apoptosis was detectable in all phases of the cell cycle (Fig. 3D). To demonstrate that this increase in apoptosis was not due to the toxicity of the thymidine, cells were also blocked in G0/G1 using one dose of 4 mM thymidine (Fig. 5). Measurement of

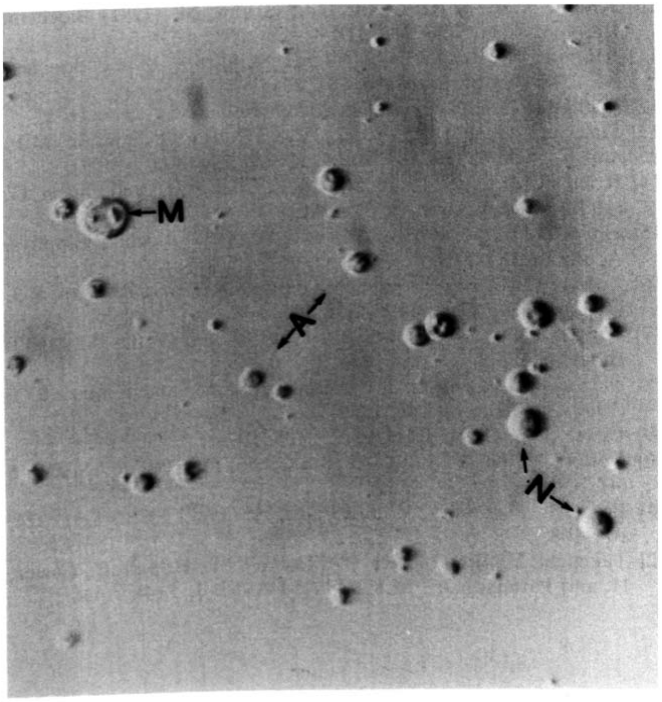

Fig. 4. Phase contrast appearance (×400) of enriched apoptotic Met B cells following fractionation on a percoll step-density gradient [18]. A, apoptotic cell; N, non-apoptotic cell; and M, cell undergoing mitosis.

apoptosis in these cells following removal of the drug indicated that the maximum apoptotic index was not found at 2 h that might be indicative of thymidine toxicity. Instead it could be shown that the greatest number of apoptotic envelopes once again correlated with the mid S-phase of the cell cycle where incorporation of [^{3}H]thymidine was at its maximum which in this experiment occurred 4 h after removal of the drug (Fig. 5).

Our results therefore indicate that in a rapidly proliferating fibrosarcoma cell line, cells undergoing spontaneous apoptosis are more likely to enter into the apoptotic programme at a specific phase of the cell cycle corresponding to that of mid S-phase. It has been suggested that failure of damaged cells to successfully pass through cell cycle check point such as G2/M may lead to apoptosis [19]. Our data indicates that this check point is likely to be in mid S-phase rather than G2/M. In keeping with this observation is the finding that agents of pharmacological nature induce death selectively in S-phase [20]. Of particular interest is the observed increase in the specific activity of transglutaminase which parallels the maximum rate of apoptosis observed during mid S-phase. Although an increase in the expression of the enzyme in the apoptotic cell has been demonstrated [6,21], no previous data has indicated a change in the specific activity of the enzyme which is indicative of a direct effect on the catalytic activity of the enzyme. The recent finding that phosphorylation of the brain transglutaminase leads to an in-

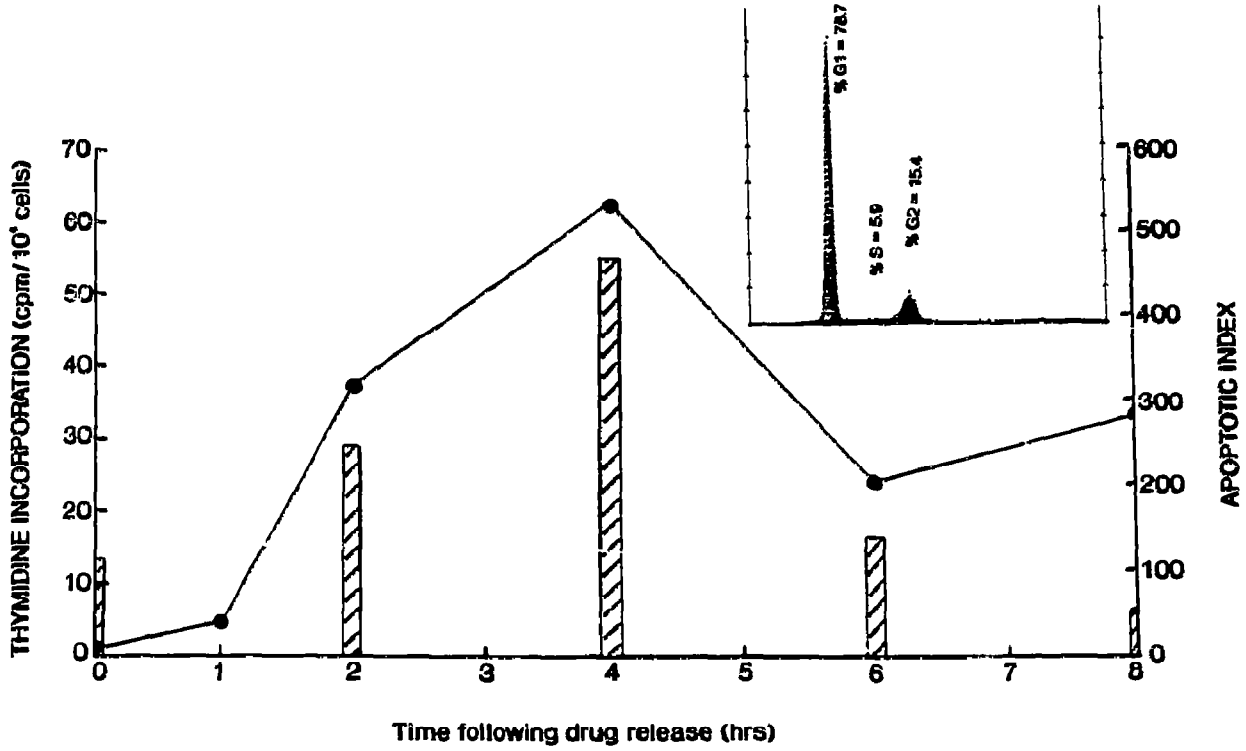

Fig. 5. DNA synthesis (line) and apoptotic index (bar) in Met B cells synchronised with one dose of 4 mM thymidine. The insert shows the DNA histogram of cells stained with propidium iodide and analysed by flow cytometry at 0 h following removal of drug.

crease in the enzymes affinity to calmodulin is particularly intriguing in this respect [22].

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