Extracellular matrix alters the relationship between tritiated thymidine incorporation and proliferation of MC3T3-E1 cells during osteogenesis *in vitro*

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Received 24 August 2000; revision accepted 9 April 2001

Abstract. Bone cells *in vivo* exist in direct contact with extracellular matrix, which regulates their basic biological processes including metabolism, development, growth and differentiation. Thus, the *in vitro* activity of cells cultured on tissue culture treated plastic could be different from the activity of cells cultured on their natural substrate. We selected MC3T3-E1 pre-osteoblastic cells to study the effect of extracellular matrix on cell proliferation because these cells undergo a progressive developmental sequence of proliferation and differentiation. MC3T3-E1 cells were cultured on plastic or plastic coated with ECM, fibronectin, collagen type I, BSA or poly L-lysine and their ability to proliferate was assessed by incorporation of [³H]dT or by enumeration of cells. Our results show that (1) ECM inhibits incorporation of [³H]dT by MC3T3-E1 cells; (2) collagen type I, but not BSA, poly L-lysine or fibronectin also inhibits incorporation of [³H]dT; (3) the level of ECM inhibition of [³H]dT incorporation is directly related to the number of cells cultured, but unrelated to the cell cycle distribution or endogenous thymidine content; (4) the kinetic profile of [³H]dT uptake suggest that ECM inhibits transport of [³H]dT from the extracellular medium, and (5) cell counts are similar in cultures whether cells are grown on plastic or ECM. These results suggest that decreased incorporation of [³H]dT by cells cultured on ECM is not reflective of bone cell proliferation.

INTRODUCTION

The MC3T3-E1 pre-osteoblastic cell line is a well-accepted model of osteogenesis *in vitro* (Quarles *et al.* 1992). Growth and differentiation factors contained in the culture medium stimulate these cells to undergo a developmental sequence that includes proliferation of undifferentiated precursors of osteoblasts which subsequently differentiate into post-mitotic osteoblasts capable of expressing the osteogenic phenotype. During the proliferative phase, these cells undergo DNA synthesis and cell division resulting in a rapid increase in cell number until the cultures become confluent. At this juncture, proliferation is downregulated and increased expression of the osteogenic phenotype is observed indicating the presence of mature osteoblasts. Osteoblasts produce

alkaline phosphatase, process procollagen to collagen, and deposit extracellular matrix containing additional proteins (e.g. osteopontin, bone sialoprotein and osteocalcin) on the substrata which is subsequently mineralized.

Extracellular matrix (ECM) serves as a repository of growth factors in the environment of osteogenic cultures (Adams & Watt 1993). It supports cells, filters soluble molecules, and can induce the osteogenic developmental sequence in pre-osteogenic cells. ECM is composed of glycoproteins, proteoglycans, and glycoaminoglycans, all of which are organized into a functional network for the accommodation of cells (Haralson & Hassell 1995). Matrix regulation of cell behaviour depends upon its composition, ability to act synergistically with growth factors and ability to engage receptors on the cell surface. Through these mechanisms cells are able to rearrange their cytoskeleton, change shape, and initiate signal transduction responses which may lead to gene expression involved in growth and function. Since proliferation precedes differentiation it would be expected that the earliest effect of ECM on the osteogenic process would be seen during the proliferative phase when pre-osteogenic cells are cultured on ECM produced by osteoblasts.

Incorporation of tritiated thymidine [^3H]dT has been used as a tool to assess the rate of DNA synthesis in cell cultures for many years (Zeigler *et al.* 1974; Ahern *et al.* 1976; Peterson 1987). Generally, cells are cultured in plastic microplates, pulse labelled with [^3H]dT, and subsequently harvested onto glass fibre filter discs. The filters are processed by washing with phosphate buffered saline (PBS), trichloroacetic acid (TCA) and ethanol and finally the counts per minute (c.p.m.) assessed in a scintillation counter. Under these conditions there is a positive linear relationship between c.p.m. and cell number per culture well. The benefit of this method is that cell cultures can be harvested semiautomatically onto filter discs in an efficient manner and the samples can be counted economically because only a small volume of scintillation fluid is required. The nucleoside enters the cell via the salvage pathway, which is mediated by facilitated diffusion (Kornberg & Baker 1992; Peters & Hausen 1971a,b; Wedner & Parker 1976). Since ECM can regulate cellular metabolism (Meredith *et al.* 1996), it is possible that incorporation of [^3H]dT may be altered when pre-osteogenic cells are cultured on ECM produced by osteoblasts.

We present evidence to show that incorporation of [^3H]dT by MC3T3-E1 pre-osteoblastic cells cultured on ECM is not reflective of cell proliferation.

MATERIALS AND METHODS

Cell cultures

MC3T3-E1 cells were obtained as a gift of Dr Gideon Rodan (Merck, Sharp and Dohme, West Point, PA, USA). Standard conditions for propagating these cells included passage every 3–4 days after release with $1\times$ trypsin–EDTA solution (0.5 g porcine trypsin and 0.2 g EDTA $\cdot$ 4 Na/L Hank's balanced salt solution (HBSS; Sigma Chemical Co., St Louis, MO, USA) (Peterson & Yamaguchi 1996). Cells were grown in T flasks with a 25-cm² growth area and they were fed with standard culture medium (minimum essential medium, alpha modification (Sigma), 2 mM L-glutamine (Sigma), 50 $\mu\text{g}/\text{mL}$ gentamicin sulphate (Sigma) and 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT, USA). Cultures were maintained in a humidified incubator in a mixture of 95% air and 5% CO₂ at 37 °C. Experimental cultures were established in either 12-well plates (3.88 cm² growth area) or 96-well plates (0.32 cm² growth area, Microtest III, Falcon, Becton Dickinson Co., Lincoln Park, NJ, USA) in the presence or absence of ECM. All plastic plates were made of polystyrene and were tissue culture treated.

Cell number and viability

Cells were enumerated in wells of both types of plates. Medium from each well was removed, the monolayers of cells were washed twice with PBS, and the cells released with trypsin-EDTA solution. A minimum of triplicate cultures was assessed for each treatment group when 12-well plates were used. In the case of 96-well plates, six wells were pooled to make one sample and the number of cells per cm² was calculated. Cell numbers were determined by haemocytometer counts and viability by trypan blue dye exclusion assay.

Flow cytometry

Flow cytometry was performed employing a fluorescence activated cell sorter from Becton Dickinson Immunocytometry Systems (BDIS; San Jose, CA), model FACSVantage™ SE. The instrument is equipped with an INNOVA™ Enterprise™ argon ion laser (Coherent, Santa Clara, CA) with ultraviolet and 488 nm wavelength outputs. For the studies reported, the 488 nm output was employed as the primary light source. Fluidics for the system was purchased from BDIS. Operation of the flow cytometer was controlled with an Apple Macintosh computer with CELLQuest software.

Cells for DNA analysis were fixed with methanol and the DNA stained with propidium iodide as previously described by Dr Anne Hurley (Becton Dickinson Immunocytometry Systems, San Jose, CA). Briefly, cells (approximately 1 million per ml) in a 1-mL volume were mixed by drop-wise addition of 2 mL of cold methanol while mixing the cells on a vortex mixer. The cells in methanol were stored in a refrigerator at 4 °C for overnight (note, 30 min is minimum fixation time). On the day of analysis, the cell suspension was centrifuged, the methanol layer removed and 500 µL of a stain solution containing propidium iodide (100 µg/mL), Triton X-100 (0.1%, v/v), EDTA (37 µg/mL) in PBS followed by 500 µL of a ribonuclease solution (2 mg/mL in PBS). The samples were incubated in the dark at room temperature for 30 min, and then placed in an ice bath and analysed by flow cytometry within 1 h, employing a FACSVantage™ SE instrument and CELLQuest software. DNA analysis was performed employing the version 2.0 ModFit LT software (Verity Software House, Inc., ME) with doublet correction through windowing.

Analysis of the intracellular thymidine content

Thymidine was assayed using a high performance liquid chromatography (HPLC) procedure developed in our laboratory. For analysis, cells washed with PBS were counted and an aliquot containing at least 1 million cells centrifuged. The supernatant was discarded and the cell pellet was extracted with 200 µL of a 0.2-M perchloric acid reagent containing 100 µM EDTA, 100 µM sodium metabisulphate and 1 µg/mL of adenosine as an internal standard. Tests showed the endogenous levels of adenosine to be about 1 ng per million cells. The amount of adenosine added to the extraction reagent was used to correct for fluid trapped in the cell pellets. The amount 100 times greater than endogenous amounts of adenosine would not significantly alter the measured peak areas for the adenosine used as internal standard. The extract was centrifuged and 100 µL of the clear supernatant were analysed by HPLC employing a stainless steel Biophase ODS column (0.46 × 25 cm; 5 µm beads; BioAnalytic Systems Inc., West Lafayette, IN), a Spectra Physics pump and controller (model SP8700; San Jose, CA). Nucleosides were detected employing an Applied Biosystem 785 A programmable absorbance detector set at a wavelength of 254 nm (Foster City, CA) and eluted peaks were quantified with a Shimatzu Integrator (model C-R3A, Kyoto, Japan). The pump flow rate was 0.75 mL/min. and column temperature was 31 °C. Two solvents were employed to elute the compounds of interest: solvent A: 0.1 M potassium phosphate, pH 7.0 and solvent B: absolute methanol. A timed gradient program was used to mix the two solvents according to the following schedule: Following equilibration of the column with

solvent A, sample (100 μ L) was injected with initiation of the following solvent program: from 0 to 5 min, gradient from 100% solvent A to 95% A plus 5% B; from 5 to 15 min, gradient to 88% A and 12% B; from 15 to 17 min, gradient to 40% A and 60% B; from 17 to 25 min, hold solvent mixture at 40% A and 60% B; and from 25 to 26 min, the gradient was reversed to return the solvent back to 100% solvent A. A minimum time period of 20 min was required to allow re-equilibration of the column with solvent A prior to injection of the next sample. Employing the procedure developed, the retention time for cytosine was 5.85 min, 9.2 min for uridine, 12.9 min for thymine, 13.2 min for thymidine and 14.6 min for adenosine. Calibration experiments showed a linear relation between volume injected and peak area, and a linear relation between peak area and amount injected from 0 to 50 ng ($r^2 = 0.9969$) for thymidine and a linear range from 0 to 100 ng ($r^2 = 0.9867$) for adenosine. The percent variance for repeated injections of thymidine was 3% and 5% for adenosine.

Preparation of ECM

Cells (5×10^3 , 10^5 or $4 \times 10^5/\text{cm}^2$) were seeded in 96-well or 12-well plates and cultured for various intervals. Wells containing medium only were used as medium treated control wells. Medium was removed from each well and the monolayers were washed twice with PBS. Cells in the monolayers were lysed by adding 0.05 M ammonium hydroxide (0.2 mL to 0.6 mL per well) (Guenther & Fleisch 1990). The plates were incubated in a CO_2 incubator for 30 min. The ECM in each well was washed twice with PBS and dried at 37 °C under sterile conditions. ECM and medium treated control wells were made ready for culture by re-equilibrating them with medium before the addition of cells.

Preparation of plastic wells coated with bovine serum albumin (BSA), poly L-lysine (PLL), or matrix proteins

BSA was prepared in PBS and added to wells in a 96-well plate. Plates were incubated at room temperature for 1 h, BSA was removed, the wells washed three times with PBS and seeded with cells ($5 \times 10^3/\text{cm}^2$) in standard culture medium.

PLL (Sigma) was added to wells ($3.91 \mu\text{g}/\text{cm}^2$) in 96-well plates and the plates incubated at room temperature (temp) for 5 min. Subsequently, the plates were washed 2 $\times$ with distilled deionized H_2O and air dried at room temp before cells were added to the wells.

Fibronectin (Fn, Sigma) was added to wells (75 or $150 \mu\text{g}/\text{cm}^2$) in a 96-well plate and the plates incubated overnight at 4 °C. The next day the wells were washed 3 times with PBS and seeded with cells ($5 \times 10^3/\text{cm}^2$) in standard culture medium.

Type I Collagen (Col, Becton Dickinson Labware, Two Oak Park, Bedford, MA) was diluted in 0.02 N acetic acid and added to wells in 96-well culture plates. The plates were incubated at room temp. for 1 h, washed 2 times with PBS, dried at room temp and re-equilibrated with medium. MC3T3-E1 cells in standard culture medium were added to the wells.

Cultures were incubated at 37 °C for 1–5 days, pulsed with [^3H]dT, harvested onto discs and processed for scintillation counting.

Uptake, and incorporation of triitated thymidine [^3H]dT

Cultures established in 96-well plastic plates were used to measure [^3H]dT uptake, and incorporation. For general incorporation studies, six cultures in a row were pulsed with [^3H]dT (0.4 μCi per well; specific activity, 6.7 Ci per mm; New England Nuclear Co., Boston, MA, USA) (Peterson 1987). Another six culture wells in the same row were not pulsed and served as controls. Plates were incubated an additional 16 h before the cultures were processed for scintillation counting. Medium from each well was removed, the cell layers washed three times with PBS and the cells

released with trypsin-EDTA solution. An automatic cell culture harvesting apparatus (Brandel, Rockville, MD, USA) was used to harvest cultures onto glass fibre filters. Sample filters were washed twice with 5% TCA, twice with absolute ethanol, placed in mini poly q vials (Beckman), dried and covered with 1 mL of scintillation fluid (Scintiverse II, Fisher, Tustin, CA, USA). The vials were capped and counted in a liquid scintillation counter (LS-5801, Beckman Instruments Inc., Fullerton, CA, USA).

For [^3H]dT uptake studies, cultures in 96-well plates were incubated for 72 h and pulsed at hourly intervals for 6 h before processing began. Culture fluid was removed and assessed for [^3H]dT activity. The wells were washed 3 times with PBS and the adherent cells treated with cold 10% TCA for 5 min. The lysate was collected along with one TCA wash, neutralized with NaOH and assessed for [^3H]dT activity. The wells were washed once with cold absolute ETOH and the plates dried at 37 °C. NaOH was added to the wells and the residues allowed to digest for 45 min at 37 °C. Samples were collected, neutralized with HCl and assessed for [^3H]dT activity. The activity of radioactive samples was assessed by placing samples in vials, adding scintillation fluid (Scintisafe 30%, Fisher) to the vials, capping and counting them in a scintillation counter.

Statistical analysis

Cell count data were expressed as the mean $\pm$ the standard error of the mean (SEM). c.p.m. data were processed by log transformation (Zeigler *et al.* 1974) and the results reported as the mean $\pm$ the standard error of the mean. The statistical significance was determined by applying a two-tailed student's *t*-test or ANOVA.

RESULTS

Effect of ECM on incorporation of [^3H]dT

In a previous study (Peterson & Yamaguchi 1996) we showed that diminished incorporation of [^3H]dT in cultures of MC3T3-E1 cells occurred at a time (post 6 days of culture) when ECM is expected to be deposited on the surface of plastic culture wells. Therefore, a study was initiated to determine whether this observation was due to a cause and effect relationship. Incorporation of [^3H]dT was assessed in cells cultured on ECM prepared from 10-day cultures (Fig. 1). It can be seen that a 6-fold decrease in c.p.m. was observed in cultures containing ECM. The results of other experiments demonstrated that this decrease in c.p.m. is related to the density of cells used to establish cultures and the time of ECM preparation after establishment of cultures. These observations suggest that incorporation of [^3H]dT in cultures of MC3T3-E1 cells may be altered by ECM.

Effect of PLL or BSA on incorporation of [^3H]dT

It is possible that decreased incorporation of [^3H]dT by cells cultured on ECM is a non-specific effect that would be observed when any material coats the plastic surface of culture wells. To test this possibility, we assessed incorporation of [^3H]dT by cells cultured in PLL- or BSA-coated culture wells. The results, Table 1 show no effect of BSA or PLL on the incorporation of [^3H]dT suggesting that if specificity exists it may be related only to components of ECM.

Effect of Fn on incorporation of [^3H]dT

We proceeded to assess incorporation of [^3H]dT by MC3T3-E1 cells cultured in wells coated with Fn, a known component of ECM. The results in Table 1 show a slight increase in incorporation

Figure 1. Decreased incorporation of [^3H]dT by MC3T3-E1 cells cultured on ECM. ECM was prepared from 10-day cultures, re-equilibrated with medium and reseeded with 5×10^3 cells per cm^2 . Incorporation of [^3H]dT was determined 3 days later. The cultures were pulsed with [^3H]dT and 16 h later harvested following trypsinization. □, untreated wells; ▨, medium treated wells; ▤, wells containing day 10 ECM. The data represent the mean of sextuplicate samples $\pm$ the standard error of the mean.

Table 1. Effect of Fn, BSA, or PLL on incorporation of [^3H]dT^a

Materials used for coating plastic surface							
Fn			BSA		PLL		
0	75 $\mu\text{g}/\text{cm}^2$	150 $\mu\text{g}/\text{cm}^2$	0	6 mg/cm^2	0	3.91 $\mu\text{g}/\text{cm}^2$	
58105 $\pm$ 7926	81634 $\pm$ 6538	76115 $\pm$ 5180	58105 $\pm$ 7926	59695 $\pm$ 13117	15522 $\pm$ 1087	14764 $\pm$ 738	

^aCells were seeded at $5 \times 10^3/\text{cm}^2$ and cultured for 4 days before the cultures were pulsed and harvested. The values represent the mean c.p.m. $\pm$ the standard error of the mean. The values for Fn at the 75 $\mu\text{g}/\text{cm}^2$ level was marginally significant, $P > 0.056$. The values for BSA and PLL were not significant ($P > 0.83$).

of [^3H]dT in Fn coated wells. Thus, decreased incorporation of [^3H]dT by MC3T3-E1 cells cultured on ECM may be associated with other ECM proteins.

Effect of Col on incorporation of [^3H]dT

Since Col is the predominant ECM protein, we next assessed its effect on incorporation of [^3H]dT by MC3T3-E1 cells. The results in Fig. 2 show decreased incorporation of [^3H]dT at the 300- $\mu\text{g}/\text{cm}^2$ level for the first 4 days and at the 75- $\mu\text{g}/\text{cm}^2$ level and the 150- $\mu\text{g}/\text{cm}^2$ level on days 4 and 5, respectively. These results suggest that Col may contribute to the decreased incorporation of [^3H]dT by ECM.

Figure 2. Effect of Col on incorporation of [^3H]dT by MC3T3-E1 cells. Wells in 96-well plates were coated with various concentration of Col and seeded with 5×10^3 cells per cm^2 . Incorporation of [^3H]dT was assessed 1–5 days later as described in Fig. 1. The concentration ($\mu\text{g}/\text{cm}^2$) of Col includes: $\square$, $\circ$, diagonal lines , 75; cross-hatch , 150; vertical lines , 300. *, Significance ($P < 0.05$).

Effect of ECM, Col, Fn, BSA, or PLL on cell proliferation

Since incorporation of [^3H]dT is an accepted method for assessing DNA synthesis and cell proliferation, it would be expected that when cells are cultured on ECM, parallel results would be obtained in assessment of cell number and c.p.m. Thus in ECM cultures, decreased c.p.m. would predict diminished cell counts. Accordingly, we first cultured cells on plastic or on ECM produced on day 7 in cultures initially seeded with $5 \times 10^3/\text{cm}^2$ or $10^5/\text{cm}^2$. In a second experiment cells were cultured on Col, Fn, BSA, or PLL. With the exception of a small decrease in cell number on days 3 and 5 after reseeding of cells, there is no decrease in cell number in cultures containing ECM (Fig. 3). There is a slight increase in the number of cells in wells containing Fn and BSA, but no increase in wells containing Col and PLL (Table 2). These results show that cells grow equally well on plastic, ECM, Col, and PLL and slightly better on Fn and BSA suggesting that decreased incorporation of [^3H]dT in cells cultured on ECM is not reflective of cell proliferation.

Analysis of the relationship between cell number and incorporation of [^3H]dT in cells cultured on ECM

We next determined whether a direct relationship exists between cell number and incorporation of [^3H]dT. The number of cells ranged from 3.1×10^2 – $9.92 \times 10^3/\text{cm}^2$ and the incorporation of [^3H]dT was measured on day 1 of culture (Fig. 4). It can be seen that a similar direct relationship exists in cultures on untreated plastic or ECM. The regression lines are parallel with a slope, intercept and coefficient of correlation of 0.926 and 0.905; 0.754 and 0.033; and 0.996 and 0.994 for cultures grown on plastic or ECM, respectively. The only difference is the magnitude of the regression lines. As expected the regression line for ECM cultures is less than the regression line

Figure 3. Effect of ECM on growth kinetics of MC3T3-E1 cells. Cultures were established in 12-well plates using 5×10^3 or 4×10^5 cells per cm^2 . ECM was prepared 7 days later, re-equilibrated with medium and reseeded with 5×10^3 cells per cm^2 . Cell counts were made at various intervals. □, cells reseeded on plastic; ▨, cells reseeded on ECM (5×10^3); ▤, cells reseeded on ECM (4×10^5). The data represent the mean of quadruplicate samples $\pm$ the standard error of the mean.

Table 2. Effect of Col, Fn, BSA or PLL on cell number^a

Materials used for coating plastic surface				
None	Col 50 $\mu\text{g}/\text{cm}^2$	Fn 75 $\mu\text{g}/\text{cm}^2$	BSA 6 mg/cm^2	PLL 3.91 $\mu\text{g}/\text{cm}^2$
402 $\pm$ 30		711 $\pm$ 76	557 $\pm$ 41	404 $\pm$ 26
119 $\pm$ 13	118 $\pm$ 5			

^aCells were seeded at $5 \times 10^3/\text{cm}^2$ and cultured for 4 days before harvesting by trypsinization. The values represent the mean cell number $\pm$ the standard error of the mean. The values were significantly different for Fn ($P < 0.001$) and BSA ($P < 0.05$). The value for PLL ($P > 0.05$) was not significant.

for plastic control cultures. These results suggest that the decreased incorporation of [^3H]dT observed in standard cultures on ECM is a universal effect occurring at all cell concentrations.

Effect of ECM on the cell cycle

Because ECM can regulate the behaviour and morphology of cells, we examined the effect of ECM on the distribution of cells in phases of the cell cycle (Fig. 5). It can be seen that no difference exist in the frequency distribution of cells cultured on plastic or ECM confirming previous observations (Fig. 3) that the rate of DNA synthesis is not changed in ECM cultures.

Figure 4. Relationship of log-c.p.m. versus log-cell number. Cultures were prepared in untreated wells or wells containing ECM in 96-well plates. The incorporation of [³H]dT was assessed 1 day later. The number of cells $\times 10^3$ seeded per cm² includes 0.3; 0.62; 1.24; 2.48; 4.96; 9.92. Cultures were processed as described in Fig. 1. ●, Cells seeded on plastic; ○, cells seeded on ECM.

Figure 5. Effect of ECM on the cell cycle distribution. Wells containing ECM were prepared and reseeded with 5×10^3 cells per cm² as described in Fig. 3. On day 3 the cells from wells of a single plate were released, collected, pooled and treated as 1 sample. Cells from each sample were stained and analysed by FACS. ▨, Cells seeded on plastic; ▤, cells seeded on ECM. The data represent the mean $\pm$ the range.

Effect of ECM on thymidine content

To further examine mechanisms that may explain decreased incorporation of [³H]dT in ECM cultures, we assessed thymidine content (Fig. 6). No difference was observed in incorporation of [³H]dT by cells cultured on plastic or ECM. This observation suggests that ECM-induced alteration of thymidine specific activity is not a factor to be considered.

Figure 6. Effect of ECM on the intracellular thymidine content. Cells were prepared as described in Fig. 5 and the thymidine content determined by HPLC. The data are presented as the mean $\pm$ the standard error of the mean. The sample size was six and five for cells grown on plastic and ECM, respectively.

Effect of ECM on uptake of [3 H]dT

To better understand decreased incorporation of [3 H]dT by cells cultured on ECM, a study was undertaken to determine whether ECM altered the process of facilitated diffusion in cultured cells. Kinetic studies in control cultures demonstrated a time-dependent loss of [3 H]dT activity from the culture fluid of cells cultured on plastic (Fig. 7). Furthermore, a corresponding increase in [3 H]dT was observed in the acid-soluble (Fig. 8) and acid-insoluble cellular fractions (i.e. DNA) of control cultures (Fig. 9). In contrast, ECM cultures (Figs 7–9) showed little or no decrease in [3 H]dT activity in the culture fluid, and minimal increase of [3 H]dT activity in the acid soluble and insoluble cellular fractions. These results suggest that ECM may decrease the extracellular transport of [3 H]dT into MC3T3-E1 cells.

DISCUSSION

[3 H]dT is commonly used to assess proliferation because it is an exogenous radioactive precursor that meets the requirements for short-term labelling by nucleotide biosynthesis along the salvage pathway (Ahern *et al.* 1976). These requirements include the use of precursors that enter cells in a rapid and efficient manner, that remain stable during uptake, that are rapidly converted to nucleotides, and that are incorporated specifically into DNA. Thus incorporation of [3 H]dT is a labelling method that can be effectively adapted to any cell system that involves proliferating cells.

The MC3T3-E1 cell line was used in this study to assess their growth on plastic or ECM during osteogenesis. Our results indicate that ECM markedly inhibits incorporation of [3 H]dT in all cell concentrations tested (Fig. 1 and Fig. 4). In contrast, our cell count data (Fig. 3) show that these cells grow equally well on plastic or ECM. A similar decrease in C.P.M. (Fig. 2) was associated with no change in cell count (Table 2) when cells were cultured in wells coated with Col compared to plastic. Furthermore, in one experiment comparing the behaviour of cells cultured on plastic or plastic coated with Fn, BSA, or PLL the incorporation of [3 H]dT was characterized by a slight increase in wells coated with Fn and no difference in wells coated with BSA

Figure 7. Effect of ECM on the level of [^3H]dT activity in culture fluid. ECM was prepared from 7 day cultures, re-equilibrated with medium and reseeded with 5×10^3 cells per cm^2 . Uptake of [^3H]dT was determined by kinetic analysis 3 days later. $\blacklozenge$, culture fluid from untreated wells; $\blacksquare$, culture fluid from wells containing ECM; Con, culture fluid from wells without cells. The data represent the mean of sextuplicate samples $\pm$ the standard error of the mean.

and PLL (Table 1). With regards to cell counts, only a slight increase in number was observed in wells containing Fn or BSA. This discordance between actual cell counts and incorporation of [^3H]dT by cells cultured on ECM, Col, Fn, or BSA suggests that incorporation of [^3H]dT is not reflective of cell proliferation.

It is conceivable that culturing MC3T3-E1 cells on ECM may alter the proportion of cells in phases of the cell cycle when compared to cells cultured on plastic. Thus, decreased [^3H]dT labelling of cells would be observed if ECM increased the proportion of cells in G_0/G_1 and/or $G_2 + M$ and simultaneously decreased the proportion of cells in S phase. However, our data (Fig. 5) show no change in the proportion of cells in the phases of the cell cycle when they are cultured on ECM or plastic, thus eliminating this possibility.

Other investigators have also demonstrated a lack of correlation between cell counts and DNA content and uptake of [^3H]dT using different cells under various culture conditions (Maurer

Figure 8. Effect of ECM on ^3H dT activity in the acid soluble cellular fraction. This is the second part of a three-part study described in Fig. 7. Culture fluid was removed and the wells washed $3\times$ with PBS before the adherent cells were treated with TCA and the acid-soluble fraction collected and the ^3H dT activity measured. $\blacklozenge$, untreated wells; $\blacksquare$, wells containing ECM; Con, wells without cells. The data represent the mean of sextuplicate samples $\pm$ the standard error of the mean.

1981). However, only a few studies have addressed the effect of ECM and its component proteins on incorporation of ^3H dT (Woodley *et al.* 1990; Greco & Ehrlich 1992). As far as we know, our study is unique in demonstrating inhibition of ^3H dT incorporation by pre-osteoblasts cultured on ECM or Col.

Col (Fig. 2), but not BSA, PLL or Fn (Table 1) inhibit incorporation of ^3H dT. These results reflect the specificity of this effect and emphasize the independent activity of individual components of a complex substrate such as ECM.

Many factors other than the proportion of proliferating cells determine the incorporation of ^3H dT. Some of these factors include; (a) the content of endogenous thymidine; (b) carriers that transport ^3H dT across the cell membrane by facilitated diffusion; and (c) enzymes that phosphorylate ^3H dT or incorporate deoxythymidinetriphosphate into DNA.

It is possible that the endogenous content of thymidine could be increased in cells cultured on ECM compared to plastic. The effect would be decreased specific activity of thymidine, which could lead to decreased incorporation of ^3H dT. However, this outcome is unlikely since our data (Fig. 6) show no difference in the endogenous content of thymidine when cells are cultured on ECM vs. plastic.

Like some nutrients, thymidine enters the cell by facilitated diffusion, meaning that it is transported into cells by a carrier (Maurer 1981). It is known that uptake of ^3H dT can be inhibited (Davison *et al.* 1979; Pfeffer & Tamm 1984) or stimulated (Woodcock & Yang 1982; Jozan *et al.* 1985) in various cellular systems depending upon the experimental agent used. In the present study we provide additional evidence that uptake of ^3H dT can be inhibited when cells

Figure 9. Effect of ECM on ^3H dT activity in the acid insoluble cellular fraction. This is the third part of a three-part study described in Figs 7 and 8. After TCA treatment described in Fig. 8 the wells were washed with cold ETOH and the adherent residue digested in 1N NaOH. The digest was assessed for ^3H dT activity. $\blacklozenge$, untreated wells; $\blacksquare$, wells containing ECM; Con, wells without cells. The data represent the mean of sextuplicate samples $\pm$ the standard error of the mean.

are cultured on ECM or Col. The mechanism of this process is not fully known, however, the kinetic profile suggests that ECM inhibits uptake of ^3H dT in a time-dependent sequence of events [depletion of ^3H dT from the culture medium (Fig. 7), appearance of ^3H dT in the TCA soluble fraction (Fig. 8), followed by appearance of ^3H dT in the TCA insoluble fraction (Fig. 9)]. Since the sequence of events of uptake of ^3H dT in ECM cultures parallel control cultures where cells are cultured on plastic, it is likely that inhibition of ^3H dT uptake by ECM is due to decreased transport of ^3H dT.

ACKNOWLEDGEMENT

This work was supported by Veterans Administration Medical Research Funds.

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