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THYMIDINE SECRETION BY CULTURED CHICKEN EMBRYO FIBROBLASTS AND NIH/3T3 CELLS: QUANTIFICATION AND TIME COURSE

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Cultured chicken embryo fibroblasts (CEF) as well as mouse NIH/3T3 fibroblasts secrete substantial amounts of thymidine into the medium. The rate of secretion is maximal when the cells become confluent. In the medium of confluent primary cultures of CEF up to 10 µM thymidine was measured. Lower concentrations (1 to 2 µM) were found in media of NIH/3T3 cultures. In media conditioned by NIH/3T3 cells, the degradation product thymine was also found, which was not detectable in the sera and the media of CEF. Moderate inhibitory effects on the growth of CEF and NIH/3T3 cells (at least 10% with a relatively large range of variation) indicated that thymidine (2 to 5 µM) might contribute to cellular homeostasis.
The secretion of high amounts of endogenous thymidine into cell culture media may falsify the assessment of DNA synthesis with labeled thymidine; thus a change of the medium is recommended when performing a thymidine pulse.

During the search for a low molecular weight growth inhibitory substance in conditioned medium of chicken embryo fibroblasts (CEF) (1) we realized that the concentration of secreted dThd is generally underestimated in such studies. Reports on the secretion and concentration of dThd in culture medium are very scarce. Secretion of labeled pyrimidines was observed in 3T3 and 3T6 cultures labeled with [^{14}C] orotic acid (2). Secretion of dThd was increased by one oder of magnitude in dThd or deoxycytidine kinase deficient cell mutants , an observation made also with CHO cells (3). In Novikoff rat hepatoma cells a large part of puls-labeled dTTP was rapidly degraded and the label found in the medium was associated with dThd (4). Excretion of mainly uridine and cytidine, normal end products of RNA metabolism, as well as some minor identified modified and unidentified nucleosides had been found in cultures of hamster embryo fibroblasts (5). In a study on the regulation of pyrimidine deoxyribonucleotide metabolism in V79 hamster fibroblasts, the secretion of small amounts of dThd among other nucleosides had been measured in short time (h) experiments (6). Assuming a constant rate of secretion during 24 h, about 0.3 µM dThd would be expected in wells containing 3 x 10^5 cells and 0.5 ml medium. The concentration of dThd in human plasma of normal subjects and solid tumor patients varies over a range from less than 0.04 to about 1 µM (7).

Thymidine at high concentration (mM) is used since years to synchronize growing cells in the S-phase of the cell cycle (8-10). On the basis of this effect and the fact that its administration to nude mice bearing

Abbreviations: CEF, chicken embryo fibroblasts; DMEM, Dulbecco's modified Eagle's medium; dThd, thymidine; FCS, fetal calf serum; MEM, Eagle's minimal essential medium; NBCS, newborn calf serum.

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human tumor xenografts resulted in tumor regression, clinical trials with dThd had been initiated to treat cancer. These studies led to the conclusion that dThd alone does not inhibit tumor growth but might synergistically modulate the effect of cytotoxic drugs (7, 11).

In this report we describe the identification and quantification of dThd in media of cultures of chicken embryo fibroblasts and of NIH/3T3 mouse fibroblasts. As the time course of appearence of dThd in the medium suggested a role of this metabolite in cellular homeostasis, the influence of thymidine on cell growth was investigated.

MATERIALS AND METHODS

Materials – Adenosine, dThd, thymine, uridine and cytidine were from Sigma, xanthosine was from Serva and [^{3}H]dThd from Amersham.

Cell culture – Primary cultures of CEF were prepared by trypsinization of 10 days old embryos. Cells were suspended in Eagle's minimal essential medium (MEM)/HEPES (10 mM) with 10 % fetal calf serum (FCS, GIBCO), seeded in 10 ml (2.5 x 10^6 cells) cultures dishes (Corning) or 24-well (0.5 ml each) plates (NUNC) and incubated at 37° C in humified 5 % CO_2 atmosphere. Secondary cultures were obtained by passaging of 4 to 5 days old primary cultures (10 ml) and tertiary cultures by passaging of nearly confluent secondary cultures. NIH/3T3 cells (American Type Culture Collection) were cultivated in Dulbecco's modified Eagle's medium (DMEM) with 10 % newborn calf serum (NBCS,GIBCO) in humified 10 % CO_2 atmosphere. Quiescent cultures of CEF were established by complete serum depletion and those of NIH/3T3 cells by reducing serum to 0.3 %. In order to count cells in the Coulter-Counter (ZM Coultronics), cultures were trypsinized, the cells suspended in 10 ml isotonic solution and each sample counted twice.

Chromatographic analysis of dThd – Protein was first precipitated by adding 50 µl perchloric acid (70-72 %; Merck) to 450 µl of culture supernatant. After centrifugation 50 µl 0.3 M KH_2PO_4 (pH 4.6) was added to 450 µl of the supernatant, prior to its neutralization by a controlled amount (120 to 125 µl) of 4 N KOH. The precipitated $KClO_4$ was spun down and the supernatant kept on ice to remove additional $KClO_4$ before injecting 20 µl of the sample into the chromatographic system (Kontron 420/430, Nova-Pak C_{18} column, 3.9 mm x 15 cm, Waters). The procedure for isocratic elution (0.5 ml/min) was essentially adopted from Wynant's (12) and the conditions were optimized for the present purpose. Elution peaks were recorded with a dual wavelength spectrophotometer (Detector 430, Kontron) connected with an integrator (LDC, Milton Roy). As soon as shifts in the retention times were occurring, the column was washed with 80 % isopropanol for 30 min and reequilibrated with the elution buffer.

RESULTS

Identification of dThd – In order to identify and quantify dThd in cell culture supernatants, an efficient chromatographic procedure was developed which separates dThd from other nucleosides and compounds occurring in the cell culture medium (Fig. 1). In medium conditioned by fibroblasts, endogenous dThd coeluting with the authentic dThd (Fig. 2) was unequivocally identified as dThd spectroscopically and by high voltage electrophoresis. In subsequent experiments, the elution patterns of the samples were recorded both at 280 and 267 nm (λ_{max}) simultaneously. In addition to the elution time the characteristic absorption ratio of dThd (A_{280}/A_{267} = 0.65) was used to identify dThd. Peaks occasionally appearing with the same elution time but a different absorption ratio were not considered. The limit for accurate integration of the peak area was 0.3 µM. A lower detection limit could easily be achieved by fluorometric detection.

Secretion of dThd – In medium from confluent cultures of 5 to 7 days old primary CEF grown in 10 ml dishes dThd was found in concentrations between 4 and 10 µM. The upper value was confirmed by a competition assay where conditioned medium was used to compete with [^{3}H]dThd for incorporation into secondary CEF (a calibration curve was established with 0 to 10 µM cold dThd). In order to follow the time course of secretion, primary CEF of different cell densities were seeded into 24-well plates instead of 10

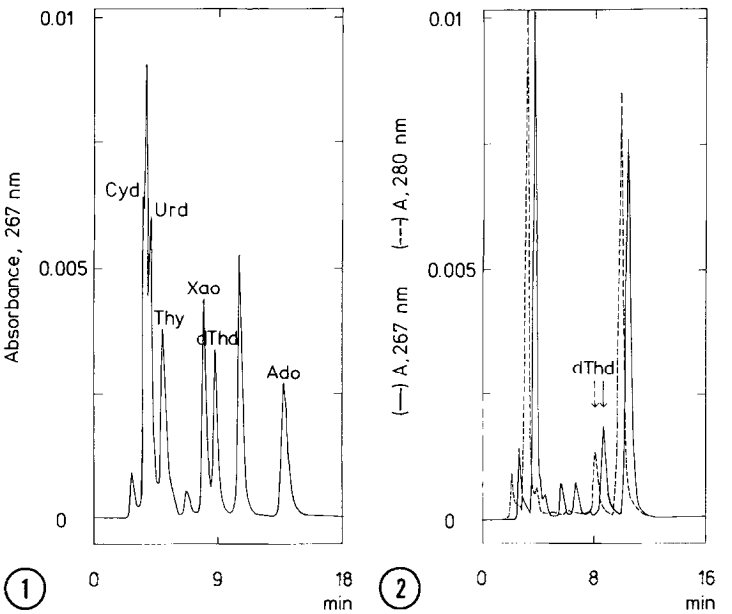

Fig. 1. Separation of a standard mixture of nucleosides and thymine by reverse phase HPLC. The compounds (10 µM each) were solved in MEM, treated as described in Materials and Methods, 20 µl injected and isocratically eluted with 30 mM KH_2PO_4 buffer (pH 4.6) and 5 % modifier (acetonitrile/ methanol, 50/50) at a flow rate of 0.5 ml/min. The following retention times were found: cytidine (Cyd) 3.7, uridine (Urd) 4.2, thymine (Thy) 5.2, xanthosine (Xao) 8.2, dThd 9.0, and adenosine (Ado) 13.9 min.

Fig. 2. A typical chromatogram of a medium conditioned by a cell culture. Primary CEF were grown for 6 days in MEM–10 % FCS, the medium collected and analyzed by HPLC (see Materials and Methods). The elution peak of dThd is indicated by arrows. The recordings of the absorbance at 267 and 280 nm are shifted against each other.

ml dishes, grown for different times, their supernatant analyzed for dThd and the cell number counted (Fig. 3). Apparently, the dThd secretion depends on cell density and appears to be maximum when the cells become confluent.

Primary cultures of CEF are not a homogeneous population and contain other cell types, e.g. myoblasts. Thus, we performed experiments with secondary (data not shown) and tertiary cultures (Fig. 4) of CEF to follow the secretion of dThd by enriched fibroblasts. A similar tendency was observed in these cultures as with primary cultures, i.e., they secret most dThd when cells have grown to a density at which they stop to proliferate. Micromolar concentrations are reached only in the confluent stage. The secretion of dThd is not restricted to CEF but NIH/3T3 cells also secrete dThd. The concentration in their media was generally lower than in those of CEF. During the first 4 days the concentration was below 0.3 µM if the cells had been seeded at a density of 1.5×10^4 cells/cm^2. In the confluent stage concentrations between 1 to 2 µM were measured. In cultures of NIH/3T3 cells dThd was degraded to thymine which was present in amounts comparable to those of dThd as shown by a peak coeluting with authentic thymine. Because dThd was still detectable in substantial amounts, the rate of its degradation must be similar or slower than that of its secretion. The degradation activity is due to the cells in culture and not to serum. When incubating dThd (10 µM) in MEM, MEM–10 % FCS, or DMEM–10% NBCS, containing each 0.4 µCi [^{3}H]dThd, at 37° C for 4, 8 and 12 days, no radioactivity could be detected coeluting with thymine, whereas [^{3}H]thymine was found in medium conditioned with NIH/3T3 cells.

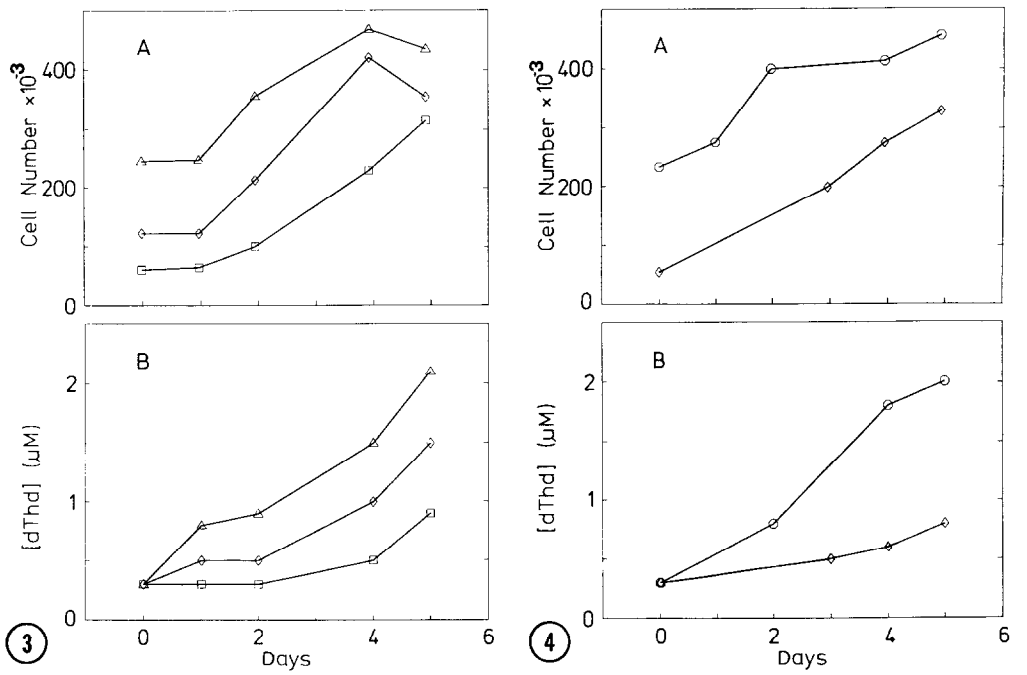

Fig. 3. Secretion of dThd in primary CEF cultures at various cell densities. 61,000 [□], 122,000 [◇] and 245,000 [△] cells/well were seeded in 24-well plates containing 0.5 ml medium. **A**: Cell numbers were determined at the indicated times. Each point represents the average of three wells. **B**: Concentration of dThd in the corresponding pooled media. The dThd concentration at time zero is contributed from the serum added.

Fig. 4. Secretion of dThd in tertiary CEF cultures. Cells were seeded at densities of 55,00 [◇] and 233,000 [o] cells/well. **A:** Triplicates were counted at the indicated times. **B:** Concentration of dThd in the corresponding pooled media.

Influence of dThd on cell growth – The secretion of dThd in micromolar range by fibroblast cells, mainly when cultures are becoming density-inhibited, suggests a role of dThd in cellular homeostasis. We investigated whether dThd influences the proliferation of fibroblasts. Thymidine was added to the medium of primary and secondary cultures of CEF as well as of NIH/3T3 cells either when cells were seeded at low, medium and high densities, or when they were made quiescent by serum depletion during one to five days, or only during the stimulation with the corresponding serum (5 %). The cell number was then counted at day 1 to 4 after stimulation. Although the different experiments under the conditions outlined above (at least three repeats) showed a relatively large variation in the proliferation behavior, a clear tendency of dThd (range 2 to 5 µM) to inhibit the growth of fibroblasts was observed which seemed not to depend on the time of addition of dThd. Inhibition was found to occur mainly during the initial phase of stimulation (primary CEF: 9 %, SEM=2 %, n=9; secondary CEF: 18 %, SEM = 4 %, n=12; NIH/3T3: 23 %, SEM=7 %, n=11; values determined after 1 d).

DISCUSSION

In the media of primary, secondary and tertiary cultures of CEF as well as NIH/3T3 cells dThd was found in concentrations up to 10 µM when cells were kept confluent. This dThd was secreted by viable cells and could not originate from DNA of dead cells as a rough calculation might illustrate. A cell contains 6.6 x 10^{-12} g DNA (13). Assuming that one fourth of it consists of thymidine, a total digestion of a confluent

culture of primary CEF with 4 x 10^5 cells in 0.5 ml would yield a concentration of 4 µM at maximum. The amounts of dThd which we have measured would suffice to double the DNA of all cells in a confluent culture. Apparently, there is no rapidly responding feedback inhibition of the synthesis of the nucleotide as indicated by the accumulation of secreted dThd over several days. Indications that the residual deoxyribonucleotide pools in density-inhibited 3T3 cells turn over at a rate comparable to that in growing cells (14) supports this conclusion. It is also in agreement with the observation that after inhibition of DNA synthesis the de novo synthesis of dCTP and TTP continued in short time (h) experiments with labeled precursor, albeit at a reduced rate, and was accompanied by secretion of deoxyribonucleosides (15). A substrate cycle constructed from a deoxyribonucleoside kinase and a deoxyribonucleotidase was suggested to contribute to the metabolism of deoxyribonucleotides in cultured cells (3, 16). Depending on the balance between the two reactions the net result of the cycle will be synthesis or degradation of the deoxyribonucleotide and favor import or export of the deoxyribonucleoside. Since thymidine kinases seem to be absent from non-growing cells (10), an increased net degradation of the deoxyribonucleotide and secretion of dThd would result as it was observed with isotope flow experiments in kinase deficient cell cultures (2, 3). Whether the substantial accumulation of dThd in the extracellular medium has a regulatory function is not known, but it seems very unlikely that we are observing a simple overflow of the nucleoside which has to be synthesized de novo by energy consuming processes. The time course of dThd appearence and its moderate inhibitory effect on growing cells points rather to a function of dThd in retaining the confluent status and might so contribute to "contact inhibition", a phenomenon still not understood.

It is possible that the local concentrations of dThd in the cell layers are higher than the concentrations measured in the medium. Thymidine at much higher concentrations (10 mM) arrested fibroblasts (not shown) and is used to synchronize cells (8-10). Ribonucleotide reductase plays a key role in the synthesis of deoxyribonucleotides. It reduces all 4 ribonucleoside diphosphates and its activity is regulated by complex allosteric modulations (16) which are thought to explain the blocking of DNA synthesis by millimolar dThd. High concentrations of dThd in the medium elevate the intracellular pool of dTTP which then could inhibit the reduction of CDP and formation of dCTP, a prerequisite for DNA synthesis (7, 10). Another possible functional role of secreted dThd in the medium could be that it affects only the stimulation by a specific locallly restricted growth factor but is not capable to inhibit the entire stimulative set of sera.

Although the role of dThd secretion remains to be established, the high amounts of dThd in the media has for sure experimental consequences. DNA synthesis and cell proliferation are routinely measured by [^{3}H]dThd incorporation. When using this procedure, endogeneous dThd will have to be taken into consideration as the amount of incorporated [^{3}H]dThd may be underestimated provoking a dramatic pseudo-inhibition of DNA synthesis. Thus, a change of the medium is recommended when a dThd pulse is performed preferably in serum-free medium, because even various amounts or different batches of sera in which we usually measured about 0.3 µM dThd will also affect the reproducibility of the experiments. Another cause for a decreased incorporation of[^{3}H]dThd might result from the cleavage of dThd to thymine as indeed was observed in the media of the NIH/3T3 cells. Such a reaction was reported to be catalyzed, e.g., by several components of human blood in particular lymphocytes (17) and is assumed to be due to a nucleoside phosphorylase.

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