Metallothionein accretion in human hepatic cells is linked to cellular proliferation

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The basal amounts of metallothionein (MT) and its rates of biosynthesis were compared in resting and proliferating Chang liver (CCl-13) cells. In resting cells the total amounts of the detectable isoforms MT-2 and MT-1e were approx. 1.6×10^6 and 4×10^5 molecules per cell respectively. In exponentially growing cultures the cellular contents of both isoforms increased co-ordinately approx. 4-fold and decreased again to the initial values within 48 h after entering density-mediated growth arrest. As documented for MT-2 its transient accretion was attributable to a 10-fold rise in the rate of biosynthesis of this protein during the growth phase. Measurements of the relative amounts of MT-2 mRNA indicated the occurrence of a more than 50 % increase within the first 12 h after subculturing of the cells, followed by a return to basal levels thereafter. These results denote a direct link between the programming of MT synthesis and proliferation and thus attest to a central housekeeping function of the MTs.

INTRODUCTION

Metallothioneins (MTs) are a family of low-molecular-mass proteins characterized by a high cysteine content and a high binding capacity for post-transition metal ions (reviewed in [1,2]). They are found in most animal cells, and related proteins occur also in plants and some micro-organisms. The precise biological function of the MTs is still a matter of discussion. Their strong inducibility by d^{10} metal ions has long implicated a special function in protection from toxic metal ions, and the increased production of some isoMTs on exposure to hormones, cytokins and many stress conditions has suggested their participation in cellular defence reactions [3–5]. However, their ubiquitous occurrence, their highly conserved structure and their programmed synthesis, noticeable in development, regeneration and reproduction [6–9], argue for a wider role in trace-metal homeostasis and metabolism and probably also in metal-modulated cellular regulation.

The human genome contains at least eight functional MT genes [10], some of which are expressed tissue-specifically [11,12]. Although in some tissues the MTs can be associated with substantial amounts of cadmium and copper [13], zinc remains the predominant and in many cases the sole metallic constituent under conditions of physiological metal exposure. In adult human liver the MTs constitute the most abundant zinc proteins [2].

Cell growth and proliferation depend on zinc [14,15], and the supply of this trace element is especially critical at the G_1/S transition of the cell cycle [16]. The observations that MTs are translocated from the cytosol to the nucleus during the cell cycle progression from G_0/G_1 to the early S-phase [17–20] could indicate a possible role of the MTs in nuclear processes of growth and cell division.

By employing the recently described HPLC method for the quantitative analysis of MTs in crude cell extracts [21] we have now measured and compared the contents and the rate of biosynthesis of some MT isoforms in resting and proliferating CCl-13 cells. The results revealed a substantial increase in MT synthesis and content during cell proliferation and therefore support a physiological function for MT in growth processes.

EXPERIMENTAL

Materials and chemicals

Dulbecco's modified Eagle's medium (DMEM), L-glutamine, gentamycin, amphotericin B (fungizone), foetal calf serum (FCS), trypsin/EDTA solution (10 ×) and *Taq* DNA polymerase were purchased from Gibco; cell culture dishes (10 cm in diameter) were obtained from Nunc. *Eco*RI and *Hin*dIII were purchased from Boehringer Mannheim. Ultrafree-CL ultrafiltration tubes (exclusion sizes 30 and 5 kDa) and nitrocellulose filters (Immobilon NC pure) were obtained from Millipore. The protein assay and disposable gel-filtration columns (Bio Gel P-10) were from Bio-Rad. Tris (Trizma base) and low-gelling-temperature (Type XI) agarose were purchased from Sigma; the scintillant mix (OptiPhase HiSave II) was obtained from LKB–Pharmacia. The RNeasy RNA miniprep kit and QIAshredder homogenization columns were obtained from Qiagen; the random priming kit Prime-It II was purchased from Stratagene. The HPLC column [Spheri-5 RP-18, 2.1 mm (internal diam.) × 220 mm; 5 μm particle size] was a product of Brownlee.

[^{35}S]Cysteine (specific radioactivity more than 600 Ci/mmol), [6-^{3}H]thymidine (specific radioactivity more than 10 Ci/mmol) and [α-^{32}P]dCTP (specific radioactivity more than 3000 Ci/mmol) were products of Du Pont–NEN.

All other chemicals were of analytical grade and obtained from either Merck or Fluka. All water used was obtained from an ultra-pure water system (Milli-Q Plus; Millipore).

Cell culture

Chang liver (CCl-13) cells were obtained from Flow Laboratories. DMEM cell culture media was supplemented with 2 mM glutamine, 50 mg/l gentamycin, 2.5 mg/l fungizone and 10 % (v/v) FCS. Cells were grown at 37 °C and 100 % humidity in 8 % air/CO_2 (11.5:1). Cell numbers and average cell diameters were determined with a Coulter Counter ZM provided with a Coulter Channelyzer 256 (Coulter Electronics). For growth experiments, density-arrested cells were plated out at a density of 34000 ± 4700 cells/cm^2.

Abbreviations used: CCl-13 cells, Chang liver cells; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; MT, metallothionein.

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Determination of protein content

After treatment with trypsin, cells were suspended in DMEM at 4 °C, centrifuged at 250 ***g*** for 10 min and resuspended in 1 ml of 25 mM Tris/HCl, pH 7.5. The cells were disrupted by ultrasonication (Sonifier cell disruptor B15; Branson) in 25 bursts of 1 s duration at 10–20 W. Aliquots of three different volumes were used to determine protein concentrations by the protein assay protocol. Protein concentrations were determined in five independently prepared cell homogenates.

Determination of rate of DNA synthesis

The rate of thymidine incorporation was determined as described [22]. Cells were incubated in DMEM containing 0.7 μCi/ml [6-^{3}H]thymidine, 50 μM thymidine and 5 μM deoxycytidine for 24 h before harvesting. After removal of the medium, cells were washed twice with cold PBS, four times with 5 % (w/v) trichloroacetic acid and twice with ethanol. The culture dish was air-dried and the cells were dissolved in 3.5 ml of 0.3 M NaOH at 37 °C. Aliquots of 0.5 ml were neutralized with 0.1 ml of 1.5 M HCl, mixed with 5 ml of scintillant mix and counted on a Model LS 1701 β-counter (Beckman). Thymidine incorporation was determined in four independently performed experiments.

Preparation of cell extract

The preparation of the cell extract and quantification of MT-2 by HPLC was performed as described [21]. Briefly, for each quantification of MT, two to five culture dishes were harvested in DMEM, pooled and, after determination of the total cell number, centrifuged at 250 ***g***, resuspended in 2.5 ml of 25 mM Tris/HCl, pH 7.5, homogenized by ultrasonication and centrifuged at 100000 ***g***. The supernatant was incubated for 4 min at 80 °C and centrifuged at 10000 ***g***. The resulting supernatant was adjusted with 25 mM Tris/HCl to a constant cellular solute concentration corresponding to that obtainable from a suspension containing 4.5×10^6 cells/ml. After centrifugation through an ultrafiltration tube, 1 ml of the filtrate was loaded on the HPLC column.

Quantification of MT-2 by HPLC and assessment of the apparent rate of synthesis of MT-2

MT was quantified by peak integration on a Data System 450 (Kontron Instruments) with purified human MT-2 as external standard [21].

The designation 'apparent rate of MT synthesis' is used because the calculated amounts represent the difference between total synthesis and degradation during the incubation period. Cells were pulse-labelled with 1.7 μCi of [^{35}S]cysteine/ml of DMEM for 24 h. The concentration of free cysteine in DMEM was 400 μM as specified by the manufacturer. The concentration increment of free cysteine introduced with FCS was 20 ± 10 μM. For radioactivity measurements, HPLC fractions were collected every 1 min, mixed with 5 ml of scintillant mix and counted for radioactivity (Model LS 1701, Beckmann). The amount of radioactivity recovered in the collected fractions ranged from 2200 to 200 c.p.m. The cysteine concentrations in the collected fractions were calculated with the specific radioactivity of the medium (4.1 μCi/μmol of cysteine). The synthesized MT-2 corresponds to 1/20 of the calculated cysteine concentration of the corresponding HPLC peak. Quantification and the assessment of the apparent rate of synthesis of MT-2 were repeated at least four times with independently prepared cell extracts.

Hybridization probes

The plasmid pEXPMTII was a gift from Dr. M. Kurasaki. After digestion of pEXPMTII with *Eco*RI and *Hin*dIII a 227 bp fragment, containing the human MT-IIA cDNA (hMT-II), was purified on a 1.0 % (w/v) agarose gel ([23], pp. 7.43–7.52). The fragment was amplified by 35 cycles of PCR (Thermocycler PHC-2; Techne) at an annealing temperature of 55 °C and a $MgCl_2$ concentration of 2 mM. Oligodeoxyribonucleotides (synthesized on an ABI 394 DNA Synthesizer) with the following sequences were used as primers: 1, 5′-CGTTCTTTA-CATCTGGGAGCG-3′ (complementary to the 3′ untranslated region of hMT-II); 2, 5′-ATGGATCCCAACTGCTCCTG-3′ (complementary to the anti-sense initial codon region). Amplified double-stranded DNA insert (50 ng) was used in a 100 μl reaction to synthesize a ^{32}P-labelled single-stranded DNA [24] with 200 pmol of primer 1, 6.25 μM unlabelled dCTP, 50 μCi [α-^{32}P]dCTP and 2 mM $MgCl_2$ at an annealing temperature of 40 °C. The synthesized MT-2 probe with a specific radioactivity of 7.3×10^8 c.p.m./μg of DNA was purified by gel filtration. Human β-actin cDNA was a gift from Professor W. Schaffner. A ^{32}P-labelled β-actin probe with a specific radioactivity of 1.1×10^9 c.p.m./μg was synthesized with the Prime-It II kit in accordance with the manufacturer's recommendations.

Total RNA preparation and Northern blot hybridization

Samples were collected at 0, 6, 12, 18, 24, 48 and 72 h and lysed directly on the culture dishes. Total RNA was prepared in accordance with the RNeasy purification protocols. Total RNA (10 μg) in sample buffer supplemented with 0.1 % (w/v) ethidium bromide was loaded on a 1.8 % (w/v) agarose gel containing 18 % (v/v) formaldehyde solution and separated with Mops buffer as electrolyte ([23], pp. 7.43–7.52). The RNA was blotted by capillary elution on a nitrocellulose membrane with 20 × SSC overnight and cross-linked with the nitrocellulose support by UV irradiation (Stratalinker UV 2400; Stratagene). The membrane was incubated with the ^{32}P-labelled MT-2 probe (1.2×10^7 c.p.m.) for 18 h at 42 °C in a sealed plastic bag. The membrane was washed twice for 5 min with 2 × SSC/0.1 % (w/v) SDS at ambient temperature and twice for 1 h with 1 × SSC/0.1 % (w/v) SDS at 70 °C. After autoradiography on a PhosphorImager (Molecular Dynamics) the MT-2 probe was removed from the membrane as described ([23], pp. 7.43–7.52). The membrane was incubated with the ^{32}P-labelled β-actin probe (10^7 c.p.m.) for 18 h at 42 °C and washed as described above. Autoradiography was performed on a PhosphorImager. All measurements were repeated at least three times with independently prepared cell lysates.

RESULTS

Figure 1 illustrates that in proliferating cells the two major isoforms, MT-2 and MT-1e, are increased severalfold in abundance. MT-2 was eluted at 41 min as the first fraction of a double peak. MT-1e was eluted at 44.5 min with a peak area consistently corresponding to 20–30 % of that of the MT-2 isoform.

Table 1 documents the growth of CCl-13 cells dispersed in DMEM and lists the actual cellular amounts of MT-2 at the different stages of growth as determined by integrating the area below the corresponding HPLC peak (Figure 1). Maximum rates of proliferation with a doubling time of 24 h were achieved by plating out cells from a confluent culture at a density of 34000 ± 4700 cells/cm^2. Under these conditions cellular growth slowed down after 48 h; by 96 h a state of density-mediated growth arrest was reached. MT-2 increased from the basal content present in freshly plated cells to an approx. 4-fold higher

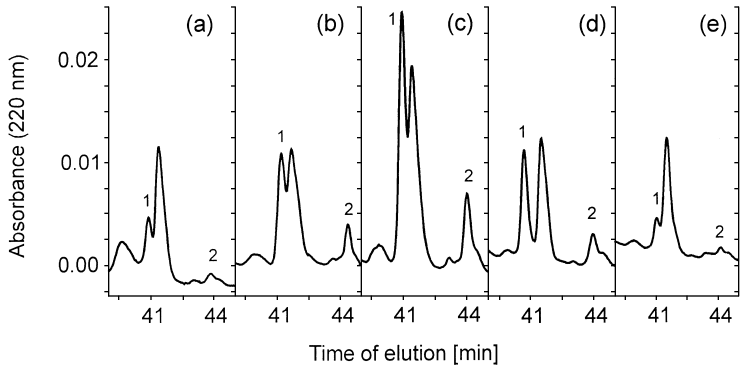

Figure 1 HPLC separation of emerging MT isoforms at successive growth stages of a CCl-13 cell culture

Confluent CCl-13 cells were plated out on 10 cm culture dishes at a density of 34000 ± 4700 cells/cm^2 and harvested in DMEM at 24 h intervals. Samples for analysis were prepared as described in the Materials and methods section. Aliquots of 1 ml were injected on a Spheri-5 RP-18 column [2.1 mm (internal diam.) $\times$ 220 mm] and eluted with a gradient formed between buffer A (25 mM Tris/HCl, pH 7.5) and buffer B [25 mM Tris/HCl, pH 7.5, containing 60% (v/v) acetonitrile] at a flow rate of 0.4 ml/min. The gradient used was as follows: 0% buffer B for 15 min; from 0 to 23% buffer B during 45 min. The effluent of the purified cell extract derived from cells harvested at 0 h (**a**), 24 h (**b**), 48 h (**c**), 72 h (**d**) and 96 h (**e**) was monitored at 220 nm. The MT isoforms are designated as follows: 1, MT-2; 2, MT-1e.

Table 1 Cell density and net cytosolic MT-2 contents at successive growth stages of cultured CCl-13 cells

Confluent cells were plated out on 10 cm culture dishes at a density of 34000 ± 4700 cells/cm^2 and allowed to grow to confluence. Cell numbers were determined every 24 h with a Coulter Counter ZM provided with a Coulter Channelyzer 256. The values shown are means $\pm$ S.E.M. for at least four independent experiments. The amount of accreted MT-2 was assessed by integration of the MT-2 HPLC peak shown in Figure 1 with purified human MT-2 as external standard. The values shown are means $\pm$ S.E.M. for four independent experiments.

Time (h)	Cell density (cells/cm^2)	MT-2 (pmol per 10^7 cells)
0	34160 ± 2129	23.4 ± 2.2
24	61620 ± 4815	66.4 ± 11.8
48	121540 ± 12570	94.3 ± 15.5
72	177600 ± 6202	52.1 ± 4.4
96	211400 ± 20820	35.5 ± 5.9

Table 2 Rates of thymidine incorporation and MT-2 synthesis at successive growth stages of cultured CCl-13 cells

Confluent CCl-13 cells were plated out on 10 cm culture dishes at a density of 34000 ± 4700 cells/cm^2 and harvested at 24 h intervals. The rate of thymidine incorporation at each time point was determined after the incubation of cells in DMEM with 0.7 Ci/ml [6-^{3}H]thymidine/50 μM thymidine and 5 μM deoxycytidine for 24 h before harvesting. After removal of the medium, the cells were washed twice with cold PBS, four times with 5% (w/v) trichloroacetic acid and twice with ethanol. After being dried, the cells were dissolved in 3.5 ml of 0.3 M NaOH at 37 °C. Aliquots of 0.5 ml were neutralized with 0.1 ml of 1.5 M HCl, mixed with 5 ml of scintillant mix and counted for radioactivity. The values are means $\pm$ S.E.M. for four independent experiments. To determine the net rate of MT-2 synthesis, [^{35}S]cysteine was added to the culture medium 24 h before harvesting. The amount of synthesized MT-2 was calculated on the basis of 20 mol of cysteine/mol of MT and a specific radioactivity of 4.1 Ci/μmol of cysteine in the medium. The results given are means $\pm$ S.E.M. for two representative, independently performed experiments.

Time (h)	[6-^{3}H]Thymidine incorporation (c.p.m./day per 10^3 cells)	MT-2 synthesis (pmol/day per 10^7 cells)
24	48.1 ± 1.1	29.5 ± 5.2
48	49.8 ± 0.7	26.1 ± 0.7
72	38.1 ± 0.8	17.2 ± 5.7
96	4.1 ± 0.6	2.5 ± 0.2

content after 48 h of growth. Beyond this time the MT-2 content declined sharply and again approached at 96 h (at growth arrest) the initial cellular content. Over the same period, from 48 to 96 h, in accord with earlier reports [25] the total amount of protein decreased moderately from 2600 ± 500 (S.D.) to 2200 ± 480 μg/10^7 cells ($n = 5$).

Table 2 shows the apparent rates of MT biosynthesis as evaluated from the incorporation of [^{35}S]cysteine into the MT-2 fraction. Paralleling the time course of DNA synthesis the rates of synthesis of MT-2, measured after a 24 h pulse with the labelled amino acid, were highest on days 1 and 2. On day 3 the rate decreased to approx. 60% and levelled off at growth arrest at less than 10% of the initial value.

The proliferation-associated stimulation of MT-2 synthesis is also reflected in a transient increase in the concentration of MT mRNA (Figure 2). Densitometric measurements of the hybridized mRNA indicated at 6–12 h of culture a 59 ± 13% increase over the level at growth arrest. Beyond 12 h, MT mRNA concentration declined sharply and at 72 h it again attained the initial concentration.

DISCUSSION

With the chromatographic method developed for this study [21] it has become possible to separate and assess the basal amounts of MT in cultured human cells and to monitor changes occurring under physiological conditions (Figure 1). Of the eight human isoMT fractions whose synthesis was enhanced by as much as 70-fold when CCl-13 cells were grown in the presence of 200 μM Zn^{2+} [26], only the two most abundantly expressed isoforms, MT-2 and MT-1e, can be detected in the unstimulated cells. Under all growth conditions examined, MT-2 was three to five times as abundant as MT-1e, suggesting co-ordinate expression of the two respective genes. Thus, for simplicity, measurements in the present study were usually restricted to the more abundant MT-2 fraction.

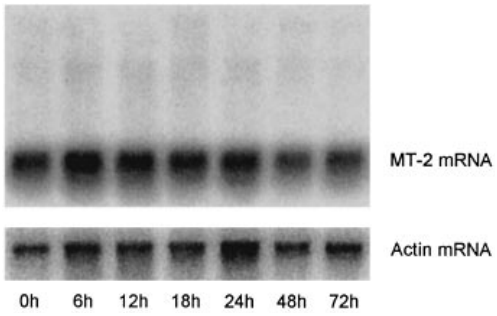

Figure 2 Northern blot analysis of MT-2 mRNA at successive growth stages of cultured CCl-13 cells

Confluent CCl-13 cells were plated out on 10 cm culture dishes at a density of $34\,000 \pm 4700$ cells/cm^2 and total RNA was prepared at 0, 6, 12, 18, 24, 48 and 72 h with the Qiagen RNeasy miniprep kit. Total RNA (10 μg) was applied to each lane of a 1.8% (w/v) agarose gel containing 18% (v/v) formaldehyde solution and separated in Mops buffer. Uniform sample loading was checked by the addition of 0.1% (w/v) ethidium bromide to the sample buffer (results not shown). RNA species were blotted on nitrocellulose with $20 \times$ SSC by capillary elution and cross-linked with the support by UV irradiation. A ^{32}P-labelled strand-specific MT-2 single-stranded DNA probe with a specific radioactivity of 7.3×10^8 c.p.m./μg synthesized in a 'run-off' polymerization with a MT-2 cDNA template was hybridized to the RNA. The MT-2 mRNA was detected and quantified with a PhosphorImager and normalized for the amount of β-actin mRNA in the samples.

For all experiments the amount of MT-2 in quiescent CCl-13 cells ranged between 21 and 28 pmol per 10^7 cells, equivalent to approx. $(1.3–1.8) \times 10^6$ molecules per cell. In actively proliferating cells MT-2 accumulated from at least 2-fold to more than 4-fold, ranging from 55 to 131 pmol per 10^7 cells or $(3.3–7.9) \times 10^6$ molecules per cell (Table 1). On the basis of an approximate cell volume of 1.7 pl in resting cells (diameter 14.8 μm) and 2.2 pl in proliferating cells (diameter 16.1 μm), this accumulation resulted in a change in the average intracellular concentration of MT-2 from 1.4 to 4.3 μM. Between the proliferating state at 48 h and the confluent state at 96 h the MT-2 concentration decreased again to less than half, whereas the concentration of total cellular protein remained unchanged.

The increase in cellular MT-2 content up to a maximum at 48 h (Table 1) is accounted for by the enhanced rate of net biosynthesis in the proliferating cells (Table 2) and coincides temporally also with the enhanced rate of DNA synthesis (Table 2). The approx. 10-fold acceleration of MT-2 production during the first 2 days of proliferation, together with the preceding transient increase of the cellular content of MT-2 mRNA (Figure 2), is consistent with an activation of transcription. The transcription of MT genes depends critically on binding of the zinc-responsive transcription factor MTF-1 to metal-responsive elements in the promoter region [27] and is stimulated by, among other factors, the interaction of the general transcription factors Sp1, AP-1 and AP-2 with basal recognition elements also located in the 5′ upstream region of the human MT-2 gene [28,29]. As the total cellular zinc content of approx. 6 nmol per 10^7 cells was not changed appreciably during cell growth (R. Studer, unpublished work) it seems most likely that the activation of the MT-2 gene is brought about by the different *trans*-acting basal recognition element-binding factors. The affinity of AP-1 for the basal recognition elements is known to be increased specifically after the activation of protein kinase C [30]; various isoforms of protein kinase C have been suggested to be positive regulators of cellular growth in liver tissue of partly hepatectomized rats [31]. Therefore we surmise that the stimulation of MT synthesis observed in this study is mediated by proliferation-linked protein kinase C-dependent signalling pathways of gene activation.

However, we do not exclude the possibility that in proliferating cells other modes of regulation such as changes in translational efficiency are also contributing to MT-2 accretion. Such accessory effects could explain why the accumulation of MT-2 continued when the level of MT-2 mRNA was already declining (Table 1 and Figure 2). Similarly, a decrease in the rate of protein degradation would be expected to enhance the steady-state content of MT-2. Preliminary pulse–chase experiments indicated that the half-life of MT-2 degradation in proliferating cells was indeed appreciably longer than in confluent cells. In the latter we found values of 21 h, which are comparable to those reported earlier for rat liver cells [32,33] and are also reflected by the time course of the decrease in cellular MT-2 content when growth arrest sets in.

Our observation that proliferating CCl-13 cells accumulate MT is in keeping with the earlier findings of a strongly increased MT content in regenerating liver of partly hepatectomized rats [34–36] and supports the histochemical evidence for the emergence of MT or MT mRNA in some tissues undergoing growth and differentiation [18,37]. The temporally limited increased synthesis accounts for the transient appearance of MT [38] and of MT mRNA in hyperplastic skin induced by exposure to tumour promoters [39–41] and for the immunohistochemical demonstration that MT is mostly expressed at the proliferating edge of malignant tumours [20]. An increase in MT content quantitatively comparable to that observed in this study was recently also noticed in the late G_1 phase and the G_1/S transition of synchronized continuously dividing human colonic cancer (HT-29) cells [42].

The transient increase in the expression of MTs in proliferating cells raises the question of their physiological function in progeneration. The relative abundance of these zinc proteins in resting cells confirmed in this study, and their further accretion with the onset of growth, argue for a role in directing the flow of zinc and in arranging its availability for the biosynthesis and signalling processes preceding and accompanying cell division. Such provisionary and regulatory functions are feasible as the binding constant for zinc in MT and in zinc-finger proteins are of comparable magnitudes and allow for a ready movement of the metal [43]. Such interchange was also demonstrated by the recently reported donation of zinc from MT to the zinc-finger-bearing nuclear oestrogen receptor [44]. Thus the transient changes in the concentration of MT and in the rate of synthesis of the zinc-accepting apoform of the MTs, as demonstrated in this study, can be conjectured to exert modulating influences on Zn^{2+}-sensitive equilibria in metabolism, gene expression and signal transduction associated with cellular proliferation [2].

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