JOURNAL OF CELLULAR PHYSIOLOGY 111:207-212 (1982)

Modulation of Synthesis of Specific Proteins in Endothelial Cells by Copper, Cadmium, and Disulfiram: An Early Response to an Angiogenic Inducer of Cell Migration

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Copper, cadmium, and disulfiram (an ionophore for copper) modulate the synthesis of several polypeptides in two clonal lines of bovine aortal endothelial cells. After treatment of Type 1 endothelial cells with 10^{-3} M $CuSO_4$ or 10^{-5} M $CdCl_2$ four cell-associated polypeptides (M_r = 28,000, 32,000, 73,000, and 83,000 daltons) were induced. In contrast, in Type 2 endothelial cells, which have cultural characteristics distinct from Type 1, only one new cell-associated protein (M_r = 32,000 daltons) was induced by $CuSO_4$ or $CdCl_2$. The same four polypeptides, described above, were induced by disulfiram (10^{-7} M) in Type 1 endothelial cells. In contrast when Type 2 endothelial cells were treated with disulfiram the synthesis of only two new cell-associated proteins (M_r = 32,000 and 40,000 daltons) was induced.

Other differences are revealed by analyses of proteins secreted into the growth medium. In particular low levels of only $CuSO_4$ (10^{-6} M) enhanced the synthesis in Type 2 cells of a protein (M_r = 220,000 daltons) identified as fibronectin. Since only copper ions induced fibronectin, we propose that the mechanism of induction of fibronectin synthesis, in contrast to the induction of cell-associated polypeptides, does not involve a sulphydryl-containing receptor molecule. It is suggested that the specific enhancement of fibronectin synthesis by copper ions may be a controlling event in the stimulation by copper ions of endothelial cell migration and angiogenesis.

The vascular endothelium consists of a monolayer of highly contact-inhibited cells at the interface between blood and other tissue. Endothelial cells mediate the transport of blood elements to the developing tissues. During vascularization, endothelial cells multiply and migrate (Ausprunk and Folkman, 1977) to form new blood vessels upon which rapid development of neoplastic tissues is dependent (Folkman, 1974). It has been postulated that vascularization can be stimulated not only by crude preparations of tumour extracts and parotid tissue (McAuslan and Hoffman, 1979) but also by chemically defined copper complexes (McAuslan, 1980). Copper salts have been shown to be the simplest angiogenic components of such tissue extracts (McAuslan, et al., 1980) and to stimulate not only angiogenesis of the anterior eye chamber in vivo but also migration of endothelial cells in vitro (McAuslan and Reilly, 1980).

Isolation of endothelial cells from bovine aorta and the establishment of cultures of endothelial cells has provided model systems for the chemical study of the angiogenic migratory process. Two morphologically and biochemically distinct lines of endothelial cells have been established (McAuslan et al., 1979; McAuslan and Reilly, 1979): Type 1, a contact inhibitable cell that forms a cobblestone monolayer characteristic of the endothelium in vivo, and Type 2, a cell line that overgrows other endothelial cells. Type 2 cells have a greater intrinsic motility than Type 1 cells in vitro and when stimulated by copper ions at 10^{-6} M deposit large amounts of the large external surface glycoprotein, fibronectin, in their migratory tracks on the culture dish (McAuslan et al., 1980). Though Type 2 cells are not easily distinguished in vivo we have proposed that they might act as lead cells in vascularization by leaving trails of fibronectin to act as a form of contact guidance for the migration of other endothelial cells and hence to establish new blood vessels (McAuslan et al., 1980). If, as we have suggested, the angiogenic process depends on the stimulation of fibronectin deposition by endothelial cells then it is of interest to determine if exogenous copper affects fibronectin synthesis by these cells. Other workers (Levinson et al., 1979, 1980) have reported that a number of sylphydryl binding agents, including copper and cadmium ions, and metal chelating drugs, including the copper ionophore disulfiram, induce the synthesis of three to four polypeptides in human and chick embryo fibroblasts. The molecular weights of the induced proteins range from M_r = 25,000 to 100,000 daltons but no biological significance has been assigned to them (Levinson et al., 1979, 1980). We

Received July 2, 1981; accepted November 23, 1981.

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0021-9541/82/1112-0207$02.00

therefore compared the effects of copper, cadmium, and disulfiram on the synthesis of proteins by endothelial cells in culture.

MATERIALS AND METHODS

Chemicals

Analytical grade copper sulphate and cadmium chloride were obtained from British Drug Houses and administered to cells as sterile filtered solutions prepared with double distilled water. Disulfiram was obtained from Sigma Chemical Co., St. Louis, MO and administered to cells as a solution in DMSO (dimethyl sulfoxide, British Drug Houses.).

Cells

Two lines of bovine aortal endothelial cells have been established (McAuslan et al, 1979; McAuslan and Reilly, 1979): Type 1, a normal parent line of contact inhibitable cells, and Type 2, a variant line of non-contact-inhibitable cells. The growth medium used was 199 (Flow Laboratories, Stanmore, N.S.W., Australia) supplemented with 10% fetal calf serum and extra folate (McAuslan et al., 1979).

Metabolic labeling

Cells were grown to high density (10^5/cm^2) in complete medium. Cells were then treated for 16 hours with 10^{-6} M $CuSO_4$ or for 3 hours with other agents and labeled with [^{35}S] methionine (New England Nuclear, Boston, MA) at 100 μCi/ml for 90 minutes in methionine-free medium (CSL). Cell medium was removed and pooled with the first wash of the monolayer and centrifuged to remove cell debris. The supernatant was made to 10% with trichloroacetic acid (TCA) and insoluble proteins centrifuged to a pellet. The cell layer was washed with serum-free medium and cells were scraped and pelleted by centrifugation. The centrifuge tubes were wiped dry and the media and cellular protein pellets solubilised in electrophoresis buffer containing 3% SDS, 62.5 mM Tris HCl, 5% β-mercaptoethanol, 10% glycerol, 2 mM PMSF, pH 6.8. Samples were heated to 100°C for 3 minutes prior to electrophoretic analysis.

Immune precipitation

Rabbit antiserum monospecific for fibronectin was a gift from Dr. Richard Hynes, Massachusetts Institute of Technology. Media from cells previously treated with drugs and radiolabeled were first incubated with the antiserum for 60 minutes at 37°C. Then goat antirabbit IgG (Dako) was added to precipitate the protein-antiserum complex. Incubation was then continued overnight at 4°C. The media samples were centrifuged and precipitates were solubilised in electrophoresis buffer. Equal volumes of extract were subjected to disc gel electrophoresis on 5% polyacrylamide gels.

Polyacrylamide gel electrophoresis

Analytical SDS polyacrylamide slab gel electrophoresis was conducted in 0.75 mm or 1.5 mm thick slab gels (Studier, 1972) composed of either 5% acrylamide stacking gel and 10% acrylamide resolving gel or 3% acrylamide stacking gel and 5% acrylamide resolving gel. Aliquots of cellular and media extracts containing equal total counts were separated in a discontinuous buffer system as described (Laemmli and Favre, 1973). The gels were then fixed, stained with Coomassie R250, dried, and exposed to x-ray films. Quantitative analyses of labeled proteins were conducted on 10 cm disc gels of polyacrylamide as described (Gordon, 1975). Disc gels were fixed and sliced into 2 mm slices and solubilised in NCS (Amersham Arlington Heights, IL) for determination of radioactivity by liquid scintillation spectrometry. Molecular weights were estimated using commercial molecular weight standards (Pharmacia South Seas, Australia).

RESULTS

Following treatment of cells with 10^{-6} M $CuSO_4$ for 16 hours or with $CuSO_4$ at 10^{-4} M or 10^{-3} M or other agents for 3 hours, cells were labeled with [^{35}S] methionine and cell-associated or secreted proteins separated according to molecular weight by SDS polyacrylamide slab or disc gel electrophoresis.

Copper, cadmium, and disulfiram enhance synthesis of specific cell associated proteins

For comparison with the data of Levinson et al. (1979, 1980), the cell-associated proteins were reduced and separated on slab gels of 10% polyacrylamide and visualised by autoradiography. As shown in Figure 1 four new cell-associated proteins (M_r = 28,000 32,000 73,000, and 83,000 daltons) were induced in Type 1 endothelial cells by $CuSO_4$ at 10^{-3} M (d), by $CdCl_2$ at 10^{-5} M (f), and by disulfiram at 10^{-7} M (g), or 10^{-6} M (h). New protein bands were barely detectable after treatment with 10^{-4} M $CuSO_4$ (b) or 10^{-6} M $CdCl_2$ (e). These proteins appear similar to those reported to be induced in chick embryo fibroblasts and human fibroblasts (Levinson et al., 1980) by the above agents. Levinson and coworkers (1979, 1980) originally estimated the molecular weights to be M_r = 100,000, 70,000, 35,000, and 25,000 daltons but more recently (Johnston et al., 1980) as M_r = 89,000, 73,000, 35,000, and 27,000 daltons. The slight difference in molecular weights of the proteins between Type 1 bovine endothelial cells and chick or human fibroblasts may be owing to species differences.

In contrast, when Type 2 bovine aortal endothelial cells were treated with the agents only two new cell-associated proteins were detected. As shown in Figure 2 treatment with $CdCl_2$ at 10^{-6} M (d) or 10^{-5} M (e), and with disulfiram at 10^{-7} M (f) or 10^{-6} M (g) induced a new protein of M_r = 32,000 daltons. Induction of the protein by $CuSO_4$ at 10^{-4} M (c) is barely perceptible. Induction was detected at 10^{-3} M $CuSO_4$ (data not shown). Type 2 endothelial cells appear to be less sensitive to induction by the agents of the four cell-associated proteins than either Type 1 endothelial cells or fibroblasts (Levinson et al., 1980). Furthermore, treatment with disulfiram at 10^{-6} M (Fig. 2g) induced a novel protein of M_r = 40,000 daltons whose significance is unknown. Treatment of Type 2 endothelial cells with $CuSO_4$ at 10^{-6} M (Fig. 2b) did not induce the synthesis of any cell-associated proteins.

Effects of copper, cadmium, and disulfiram on synthesis of large molecular weight cell associated proteins including fibronectin

During an investigation of the stimulation of migration of Type 2 endothelial cells cells by copper ions (McAuslan et al., 1980), we observed a qualitative increase in the deposition of fibronectin. We were therefore interested in the ef-

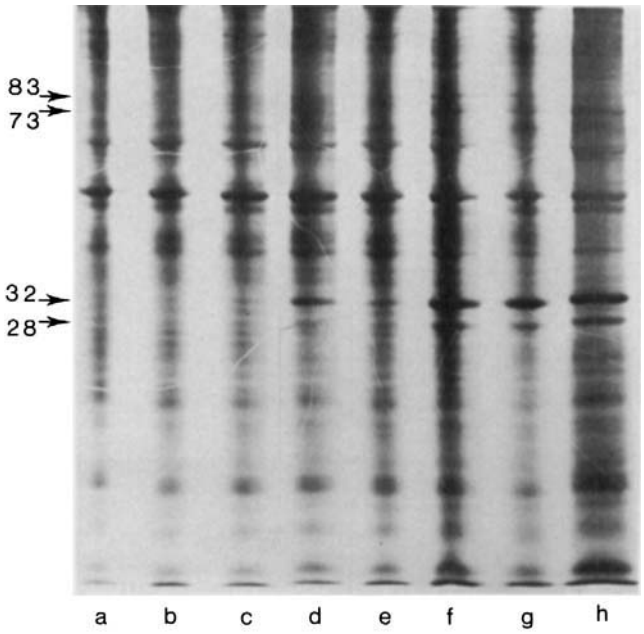

Fig. 1. Autoradiographic patterns of [^{35}S] methionine labeled cell-associated proteins from Type 1 endothelial cells separated by 10% polyacrylamide SDS gel electrophoresis. (a) No drug; (b) $CuSO_4$ 10^{-6} M; (c) $CuSO_4$ 10^{-4} M; (d) $CuSO_4$ 10^{-3} M; (e) $CdCl_2$ 10^{-6} M; (f) $CdCl_2$ 10^{-5} M; (g) disulfiram 10^{-7} M; and (h) disulfiram 10^{-6} M. Numbers in the margin represent molecular weights $\times$ 10^{-3} daltons.

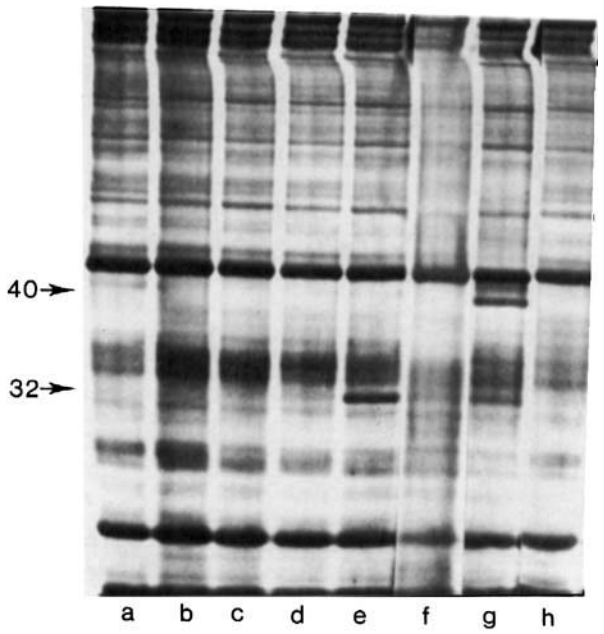

Fig. 2. Autoradiographic patterns of [^{35}S] methionine labeled cell-associated proteins from Type 2 endothelial cells separated by 10% polyacrylamide SDS gel electrophoresis. (a) No drug; (b) $CuSO_4$ 10^{-6} M; (c) $CuSO_4$ 10^{-4} M; (d) $CdCl_2$ 10^{-6} M (e) $CdCl_2$ 10^{-5} M; (f) disulfiram 10^{-7} M; (g) disulfiram 10^{-6} M; and (h) DMSO 0.1% solvent for disulfiram. Numbers in margin represent molecular weights $\times$ 10^{-3} daltons.

fect of copper and related agents on fibronectin synthesis. Fibronectin is a dimeric glycoprotein of M_r = 440,000 daltons, which upon reduction to monomers of M_r = 220,000 daltons can be detected on 5% polyacrylamide gels (Choi and Hynes, 1979). Since newly synthesised fibronectin is both secreted into the growth medium and incorporated in the cell matrix (Choi and Hynes, 1979) we investigated its presence at both sites. We considered first the cell-associated large molecular weight proteins. When the radiolabeled cell-associated proteins of Type 2 endothelial cells were reduced and separated on a 5% polyacrylamide slab gel (Fig. 3), a prominent cell product was the band at M_r = 220,000 daltons, which corresponded in electrophoretic mobility to fibronectin. Quantitative anlaysis on disc gels revealed this band to represent 5% of the total cell-associated proteins synthesised by control cells. This represented a rate of incorporation of [^{35}S] methionine of 0.7 $\times$ 10^5 cpm/hr/dish into fibronectin. However treatment of Type 2 endothelial cells with copper, cadmium, or disulfiram induced no increase in any cell associated protein in the molecular weight range M_r = 90,000 to 300,000 daltons. Similar results were obtained for Type 1 endothelial cells (data not shown). Of interest was an apparent depression by disulfiram of the synthesis of a protein M_r = 200,000 daltons for both Type 1 and Type 2 endothelial cells (Fig. 3f,g). This protein corresponds in electrophoretic mobility to laminin (Sakashita et al., 1980).

Effects of copper, cadmium, and disulfiram on synthesis of large molecular weight proteins secreted into the medium: copper ions enhance the synthesis of fibronectin

The proteins synthesised and secreted into the medium during radiolabeling with [^{35}S] methionine were separated on 5% polyacrylamide slab gels and disc gels. Presented in Figure 4 are the qualitative effects of $CuSO_4$ (b,c), $CdCl_2$ (d,e), and disulfiram (Fig. 4f,g) on the synthesis of secreted proteins, by Type 2 endothelial cells, in the molecular weight range M_r = 90,000 to 300,000 daltons. As shown, two major products of M_r = 220,000 and 160,000 daltons were synthesised and secreted by all cells except those treated with disulfiram (Fig. 4f,g). On the basis of their electrophoretic mobilities the two proteins have been identified as fibronectin and collagenous protein (McAuslan et al., 1981), respectively. Changes in synthesis of the M_r = 220,000 dalton protein owing to the agents were difficult to appreciate in a slab gel autoradiogram (Fig. 4) owing to the high background of fibronectin secreted by control cells. However when the distribution of secreted proteins was quantified by disc gel electrophoresis on 5% polyacrylamide gels, we observed that the proportion of secreted protein of M_r = 220,000 daltons was increased 300% by treatment of Type 2 endothelial cells with 10^{-6} M or 10^{-4} M $CuSO_4$. As shown in Figure 5a, the protein of M_r = 220,000 daltons represents a major portion (8%) of the total proteins secreted by control cells. This proportion represented a rate of incorporation of [^{35}S] methionine of 2.7 $\times$ 10^5 cpm/hr/dish, and when it is compared to the data for incorporation into cell-associated proteins it demonstrates that 80% of newly synthesised fibronectin was secreted to the medium by Type 2 endothelial cells. However, after treatment of Type 2 endothelial cells with 10^{-6} M $CuSO_4$ the proportion increased to 31% (Fig. 5b). This represented a rate of incorporation of [^{35}S] methionine into fibronectin of 1.4 $\times$ 10^6 cpm/hr/dish. The significance of this finding de-

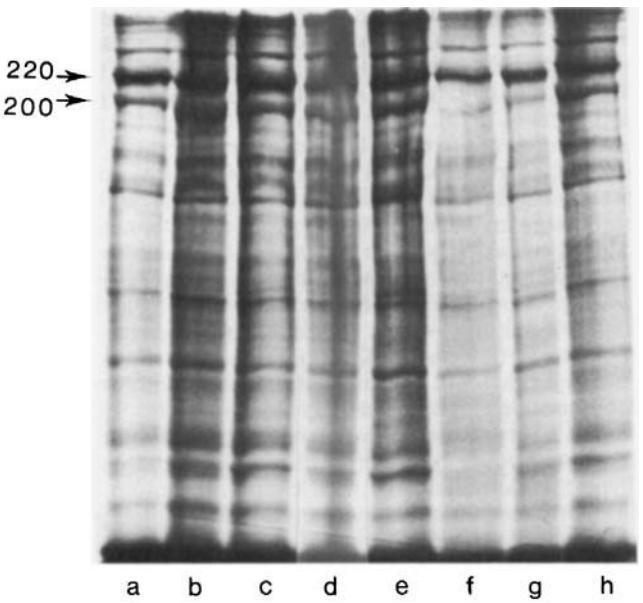

Fig. 3. Autoradiographic patterns of [^{35}S] methionine labeled cell-associated proteins from Type 2 endothelial cells separated by 5% polyacrylamide SDS gel electrophoresis. (a) No drug; (b) $CuSO_4$ 10^{-6} M; (c) $CuSO_4$ 10^{-4} M; (d) $CdCl_2$ 10^{-6} M; (e) $CdCl_2$ 10^{-5} M; (f) disulfiram 10^{-7} M; (g) disulfiram 10^{-6} M; and (h) DMSO 0.1%. Numbers in margin represent molecular weights $\times 10^{-3}$ daltons.

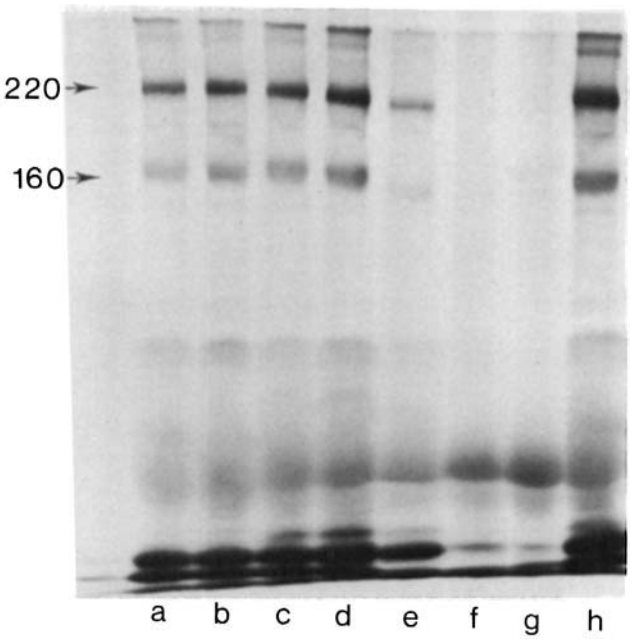

Fig. 4. Autoradiographic patterns of [^{35}S] methionine labeled proteins secreted into the medium by Type 2 endothelial cells and separated by 5% polyacrylamide SDS gel electrophoresis. (a) No drug; (b) $CuSO_4$ 10^{-6} M; (c) $CuSO_4$ 10^{-4} M; (d) $CdCl_2$ 10^{-6} M; (e) $CdCl_2$ 10^{-5} M (f) disulfiram 10^{-7} M; (g) disulfiram 10^{-6} M; and (h) DMSO 0.1%. Numbers in margin represent molecular weights $\times 10^{-3}$ daltons.

pended upon establishing the identity of the M_r = 220,000 dalton protein. Therefore the radiolabeled media of control and copper-treated Type 2 endothelial cells were subjected to double-antibody immunoprecipitation, specific for fibronectin. When the immunoprecipitates were analysed on 5% polyacrylamide disc gels, single peaks were observed at M_r = 220,000 daltons (Fig. 5c,d). A three-fold increase in the peak area was found for cells treated with 10^{-6} M $CuSO_4$ (Fig. 5d) compared to control cells (Fig. 5c). We therefore confirmed that the bulk of the protein of M_r = 220,000 daltons whose synthesis was enhanced by copper ions was immunologically identical to fibronectin.

Quantitative disc gel analyses also showed that treatment of Type 1 or Type 2 endothelial cells with disulfiram (10^{-6} M) resulted in a 95% depression of the proportions of secreted fibronectin and of the proteins of M_r = 160,000 daltons. In contrast cadmium treatment did not affect the synthesis of secreted proteins in either cell line, and treatment with copper did not enhance fibronectin synthesis in Type 1 endothelial cells.

A summary of the effects of copper, cadmium, and disulfiram on cell protein synthesis is presented in Table 1.

DISCUSSION

It has been reported (Levinson et al., 1979, 1980) that transition series metals, metal chelating drugs, and sulphydryl reagents induce a set of cell-associated proteins in human and chick embryo fibroblasts. Experiments described here demonstrate that induction of protein synthesis by these agents is not restricted to fibroblasts but also occurs in an epithelioid cell line, Type 1 bovine aortal endothelial cells. Both the chick fibroblast cells studied by Levinson et al. (1979, 1980) and our Type 1 endothelial

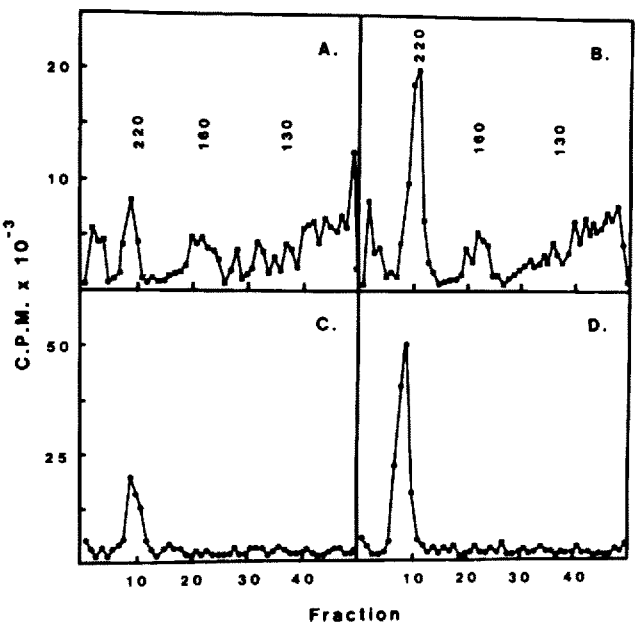

Fig. 5. Distribution of radioactivity in precipitates of proteins secreted by Type 2 endothelial cells and separated on 5% polyacrylamide SDS disc gels. TCA precipitates of medium proteins from cells treated with (A) no drug, and (B) 10^{-6} M $CuSO_4$. Fibronectin specific immunoprecipitates of medium proteins from cells treated with (C) no drug, (D) $CuSO_4$ 10^{-6} M. Fractions represent 2 mm gel slices and numbers above peaks represent molecular weights $\times 10^{-3}$ daltons.

TABLE 1. Effects of copper, cadmium, and disulfiram on protein synthesis by cultured cells

Cell type[1]	Agent	Minimun concentration[2]	Proteins[3]	Location[4]	Effect[5]
Type 1 endothelial	$CuSO_4$	10^{-3} M	28,32,73,83	CA	↑
	$CdCl_2$	10^{-5} M	28,32,73,83	CA	↑
	Disulfiram	10^{-7} M	28,32,73,83	CA	↑
	Disulfiram	10^{-7} M	220,160	E	↓
	Disulfiram	10^{-7} M	200	CA	↓
Type 2 endothelial	$CuSO_4$	10^{-6} M	Fibronectin (220)	E	↑
	$CuSO_4$	10^{-3} M	32	CA	↑
	$CdCl_2$	10^{-6} M	32	CA	↑
	Disulfiram	10^{-6} M	32,40	CA	↑
	Disulfiram	10^{-7} M	220,160	E	↓
	Disulfiram	10^{-7} M	200	CA	↓
Chick embryo Fibroblasts	$CuSO_4$	10^{-4} M	25,35,70,100	CA	↑
	$CdCl_2$	10^{-6} M	25,35,70,100	CA	↑
	Disulfiram	10^{-7} M	25,35,70,100	CA	↑

[1]Data for chick embryo fibroblasts from Levinson et al., (1979, 1980).
[2]Minimum effective concentration tested.
[3]Proteins are presented as molecular weight classes, $M_r \times 10^{-3}$ daltons.
[4]Location of proteins: CA, cell associated; E, extracellular (medium).
[5]Upward pointing arrows indicate increased synthesis and downward pointing arrows indicate decreased synthesis.

cells were induced to synthesise four proteins, in the molecular weight range M_r = 25,000 to 100,000 daltons, by copper, cadmium, or disulfiram. The fibroblasts and endothelial cells appear to share a common mechanism for protein induction and its nature can be ascertained from the following facts: (i) all four proteins arise apparently from de novo synthesis but will need to be rigorously established for these cells; (ii) cadmium and copper are sulphydryl-binding transition series metals (Levinson et al., 1980), (iii) disulfiram is an ionophore of copper (Levinson et al., 1979), and (iv) similar high concentrations of agents are required for induction in both cell lines. It is likely that the mechanism involves a sulphydryl-containing target molecule that, by nonspecific interaction with copper, cadmium, or other sulphydryl binding agents, mediates the induction of synthesis of the four novel cell-associated proteins.

The same general mechanism appears to operate in Type 2 endothelial cells for the induction of the protein M_r = 32,000 daltons. However the mechanism of induction of the protein M_r= 40,000 may be different as synthesis was induced only by disulfiram. Perhaps in this case, treatment with the copper ionophore achieves more efficient localisation of metal ions at the target than treatment with metal salts at the given concentrations. As no other cell-associated proteins were induced by copper, cadmium, or disulfiram in Type 2 endothelial cells, these cells can be distinguished from other eukaryotic cells so far studied. They may have a reduced capacity to bind and transport metal ions, by a general sulphydryl receptor mechanism, compared to other cells.

The enhancement of synthesis of secreted fibronectin is a very specific response in Type 2 endothelial cells to treatment with copper ions. It differs from the process of induction of the four cell-associated proteins in that (i) a copper ion concentration as low as 10^{-6} M initiated the enhancement of synthesis, (ii) it represents enhancement above a preexisting rate of synthesis rather than de novo synthesis, (iii) it is specific for copper ions in that no induction was detected in response to cadmium or disulfiram, and (iv) it appears to be specific for Type 2 endothelial cells. Therefore enhancement of fibronectin synthesis by copper ions does not appear to be mediated by receptor molecules with broad specificity for metal ion binding, such as sulphydryl groups (Levinson et al, 1980).

It is noteworthy that the enhancement of fibronectin synthesis also displays subcellular specificity. Although the rate of synthesis of the secreted form of fibronectin was increased, no increase in the synthesis of cell-associated fibronectin was observed after treatment of Type 2 endothelial cells with copper ions. However, when one considers that only 20% of the total fibronectin synthesised by control cells is retained at the cell surface, then it is possible that early saturation of cell surface sites for fibronectin, during copper ion treatment, might obscure any observable increase at the cell surface.

The enhancement of fibronectin synthesis by copper ions may be of physiological significance because copper ions induce the migration of cultured endothelial cells and angiogenesis in vivo (McAuslan, 1980). The increased synthesis of secreted fibronectin could account for the increased deposition of fibronectin observed (McAuslan et al., 1980) for Type 2 endothelial cells that were stimulated to migrate by copper ions.

Also noteworthy was the evidence that disulfiram inhibited the expression of several large molecular weight proteins, including secreted fibronectin and possibly laminin, in endothelial cells. A full account of the effect of disulfiram on endothelial cell protein synthesis will be presented elsewhere, but it is pertinent to the present study that disulfiram, as an ionophore of copper, might otherwise be expected to be as efficient as copper ions in enhancing fibronectin synthesis. Some other property of disulfiram must negate enhancement of synthesis of fibronectin.

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