Metallothionein response in gills of *Oreochromis mossambicus* exposed to copper in fresh water

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Dang, ZhiChao, Robert A. C. Lock, Gert Flik, and Sjoerd E. Wendelaar Bonga. Metallothionein response in gills of *Oreochromis mossambicus* exposed to copper in fresh water. *Am. J. Physiol.* 277 (*Regulatory Integrative Comp. Physiol.* 46): R320–R331, 1999.—Freshwater *Oreochromis mossambicus* (tilapia) were exposed to 3.2 μmol/l $Cu(NO_3)_2$ in the water for up to 80 days, and copper (Cu) and immunoreactive metallothionein (irMT) were localized in the branchial epithelium. Cu was demonstrated in mucous cells (MC), chloride cells (CC), pavement cells (PC), respiratory cells (RC), and basal layer cells (BLC) via autometallography combined with alcian blue staining for MC and Na^+-K^+-ATPase immunostaining for CC and, on the basis of their location in the epithelium of PC, RC, and BLC. In control fish (water with Cu concentration ≤90 nmol/l) incidentally irMT was observed in the area where progenitor cells of the branchial epithelia reside, as demonstrated by proliferating cell nuclear antigen staining. This was also the area where the first increase irMT expression of the Cu exposure was observed. After 2 days of exposure to Cu, irMT was found in CC and PC. From 5 days on, a pronounced irMT staining was observed in BLC of branchial epithelium, which then appeared to migrate and differentiate into mature CC, PC, and RC. We conclude that MT expression in mature CC, PC, and RC requires exposure to Cu in a earlier stage of development of these cells. Once expression is initiated in undifferentiated cells, MT remains expressed throughout the life cycle of the cell.

chloride cells; pavement cells; mucous cells; basal layer cells; macrophages

THE GILLS IN FISH ARE THE prime target for toxic actions of waterborne copper (Cu) (15, 17). Fish exposed to waterborne Cu accumulate this metal in their gills, and this eventually leads to damage and dysfunction of the organ (25, 30). However, in precisely which cells Cu accumulates is not known.

Fish gills represent a multifunctional organ, which carries out ion transport activities, gas exchange, acid-base regulation, and waste excretion via the branchial epithelium (40). The branchial epithelium of fish gills is complex and contains four main types of differentiated cells: pavement cells (PC), chloride cells (CC) or ionocytes, mucous cells (MC), and respiratory cells (RC) (18, 31, 40). PC form the upper layer of the filament epithelium and are characterized by microridges. Consensus exists that CC are involved in active ion transport. RC, the cells forming the lamellar epithelium, are the main site of respiratory gas exchange (32, 40). MC provide the epithelium with a protective and lubricating cover that also provides an unstirred layer (10, 35). With respect to ion transport, PC and CC may not be fully independent; Na^+ uptake across fish gills depends on H^+-ATPase activity and Na^+ channels, supposed to be predominantly located in PC, as well as on Na^+-K^+-ATPase activity, the bulk of which is located in the CC (24, 31). It is assumed that all cells of the branchial epithelium are continuously recruited from undifferentiated cells in the middle and basal layers of the filament epithelium (8, 18, 33, 37). However, there is no direct experimental evidence showing the precise origin of the branchial epithelial cells.

Cu is a rather specific inhibitor for Na^+ uptake in fish gills (17, 27) and induces necrosis and apoptosis of CC, PC, and RC (2, 23, 30). Fish appear to perceive elevated levels of Cu in the water, react to waterborne Cu with a stress response (30), and may eventually acclimate to sublethal levels of Cu (27). The acclimation is characterized by replacement of damaged cells (23, 30) and restoration of ion transport activity (17, 27). However, little experimental information is available on the mechanisms underlying the cellular restoration of branchial functions.

Branchial damage by Cu could be prevented or counteracted by an increased rate of synthesis of metal binding proteins, such as metallothionein (MT), low molecular mass (6.0–7.0 kDa) metal-binding proteins involved in the sequestration and detoxification of heavy metals, including Cu (14, 27, 29). In an earlier study on the gills of cadmium (Cd)-exposed tilapia in our laboratory, we have presented biochemical evidence for the induction of cysteine-rich proteins, with a molecular weight in the range of MT (9). Subsequently, induction by heavy metals of MT gene expression and the presence of MT have been reported for the gills of rainbow trout (12, 28). However, little is known about the mechanisms of acclimation to this metal and the dynamics of MT induction and the cellular location of these proteins in gills.

The main purpose of this study is to address the mechanism of Cu-induced MT expression in the gills. To this end, we applied autometallography (6, 36) to visualize the sites of Cu accumulation in fish gills. Furthermore, histochemical and immunocytochemical techniques were used, separately or in combination, to determine the location and the time course of the induction of MT in the different branchial cell types.

MATERIAL AND METHODS

Fish

Male and female tilapia, *Oreochromis mossambicus*, with a mean weight of 19 ± 6 g (range 12–38, $n = 82$) were obtained

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from laboratory stock. Groups of 35 fish were housed in a 200-liter aquarium provided with Nijmegen tap water at 26°C, which was continuously aerated, filtered, and recirculated by means of an Eheim pump at a rate of 600 l/h. The concentration (in mM) of the main ions in the water is 5.0 Na^+, 0.7 Ca^{2+}, 0.2 Mg^{2+}, 4.2 Cl^-, and 0.5 SO_4^{2-}. The concentration of Cu, Cd, and zinc (Zn) in the tap water used were less than 90, 0.1, and 1 nmol/l, respectively. The water pH was 7.6. Fish were fed daily with Trouvit pellets (2% of the total fish wet wt/day). The Cu and Cd contents of the food were less than 93 and 1.2 nmol/g dry food, respectively. Lights were on for 12 h per day.

Exposure to Cu

Fish were exposed to 3.2 µmol/l (~200 parts per billion) Cu as described previously (23). In brief, $Cu(NO_3)_2$ solution was infused over a 24-h period to reach the final concentration, starting with fresh water containing a basal Cu level of 90 ± 6 nmol/l (n = 40). Cu levels in water were monitored by atomic absorption spectrometry (AAS, Philips PU 9200, connected to an electron thermal atomizer, Philips PU 9390X) of samples taken daily. During the experiments no mortality was observed. The total experimental period lasted 80 days. At 2 h, and 1, 2, 5, 8, 11, 14, 19, 28, 40, and 80 days after the start of the experiment, six fish were removed from control and experimental tanks and quickly anesthetized in phenoxyethanol (Fluka Chemicals, diluted 1:3,000 in water). Sampled fish were weighed, and blood samples were collected from the caudal vessels using heparinized capillaries. Plasma, separated from the blood cells by centrifugation (3 min, 6,000 *g*), was stored at −20°C until analysis.

Cu Measurements

Individual gill arches were lyophilized to constant weight, destructed with 0.5 ml of nitric acid (65% HNO_3 ultrapur, Merck), and subsequently diluted to 0.2% HNO_3 before analysis. Water samples were acidified immediately on collection with 0.2% (vol/vol) nitric acid. Plasma samples were digested in an equivalent volume of 65% HNO_3 and subsequently diluted to 0.2% HNO_3 before analysis. Cu concentrations were estimated by AAS using a certified $Cu(NO_3)_2$ solution (Spectrosol, British Drug House) as reference.

Histology

Autometallography. Gills were fixed in Bouin's fluid for 24 h at room temperature. The autometallographic procedure was a modification of the procedure of Danscher (6) and Soto et al. (36). Briefly, paraffin sections (5 µm) were dewaxed in xylene, hydrated through an ethanol and water series, and dried in an oven at 37°C. Next the sections were covered by dipping with a uniform layer of photographic emulsion (Ilford Nuclear Emulsion K2) under safe light, dried, and then rinsed in Kodak D-19. After a 1-min wash in running water, the sections were fixed (sodium thiosulfate 1:9) for 10 min. The procedure yields silver grains at the sites of Cu deposition. Finally, the sections were washed in running water for 30 min, dehydrated, and mounted in Entellan.

Double staining of Cu with alcian blue or anti-Na^+-K^+-ATPase. To study the localization of Cu in MC, a combination of autometallography and alcian blue (AB, pH 2.5) staining was used. In brief, after autometallographic staining for Cu, sections were immersed in AB solution for 10 min and then rinsed in water, dehydrated via increasing concentrations of ethanol, and mounted in Entellan. To localize Cu in periodic acid Schiff (PAS, pH 6.2)-positive cells, adjacent sections were subjected to autometallography and PAS staining (1), respectively. The strong reducing properties of PAS staining do not allow for simultaneous autometallography.

To localize Cu in CC, autometallography was combined with immunostaining for Na^+-K^+-ATPase. In short, after autometallographic staining for Cu, slides were rinsed in two changes of 0.05 M Tris-buffered saline (150 mM NaCl containing 0.03% Triton X, pH 7.6; TBS TX) for 10 min each. Subsequently immunostaining for Na^+-K^+-ATPase was carried out and visualized by 3, 3′-diaminobenzidine (DAB) as described in *Immunocytochemistry*.

Immunocytochemistry

Light microscopic localization of MT and PCNA. MT immunocytochemistry was carried out on tissue sections of gill arches and on whole gill arches. Proliferating cell nuclear antigen (PCNA) immunostaining was applied to tissue sections. Bouin-fixed (24-h) gills were paraffin embedded, cut at 5 µm, and mounted on poly-L-lysine-coated slides (Sigma, St. Louis, MO). For whole gill arch preparations individual gill arches were fixed in 3.5% paraformaldehyde-1% glutaraldehyde in 0.4 M phosphate buffer (PB) at pH 7.4 for 2 h at room temperature. They were kept in 0.1% glutaraldehyde at 4°C until further processing, without obvious signs of loss of antigenicity. An avidin-biotin-peroxidase complex (ABC)-based method of immunocytochemical detection of MT and PCNA was chosen (13). Endogenous peroxidase was blocked by 20 min of incubation in methanol solution with 2% H_2O_2 at room temperature. Slides were then rinsed in two changes of TBS TX for 5 min each and incubated with 20% normal goat serum for 30 min. For whole mount staining, filaments were washed in distilled water and incubated in TBS TX solution with 1% (vol/vol) normal goat serum and 0.5% (wt/vol) BSA for 2 h at room temperature. A polyclonal antiserum raised in rabbit against perch (*Perca fluviatilis*) MT or mouse monoclonal antibody PCNA (clone PC10, Calbiochem, NA03) was applied at a dilution of 1:4,000 (for whole mounts 1:6,000) and incubated overnight at room temperature. Biotinylated goat anti-rabbit IgG or goat anti-mouse IgG was used as second antibody for 1 h at the room temperature and peroxidase-conjugated streptavidin (ABC kit, Vector Laboratories, prepared at least 30 min before use) for a further 1 h. Between each step the sections or tissues were washed twice for 10 min in TBS TX solution. Thereafter, DAB in TBS (0.05 M TBS, pH 7.6) with H_2O_2 (0.03%) was applied at room temperature. Finally, the sections were dehydrated and mounted. The whole mount tissues were stored in TBS solution at 4°C. In controls the first antiserum was omitted.

Double staining for Na^+-K^+-ATPase and MT. After dewaxing and blocking of endogenous peroxidase, slides were rinsed in two changes of TBS TX for 10 min each. A mouse monoclonal antibody against avian Na^+-K^+-ATPase (IgG5, Johns Hopkins University, dilution 1:100) and the aforementioned MT antibody (1:4,000) were simultaneously applied and incubated overnight at room temperature. Biotinylated anti-rabbit IgG was used as a second antiserum for MT for 1 h at room temperature. Texas Red conjugated goat anti-mouse was used to probe Na^+-K^+-ATPase, and the biotinylated anti-rabbit IgG was probed with streptavidin FITC. Incubations lasted 1 h in all cases. Between each step the sections were washed twice in TBS TX.

Ultrastructural localization of MT. After staining whole gill arches using DAB as the chromogen, the tissue was postfixed in 1% OsO_4 in PB for 1 h. After several washes in distilled water, the tissue was then dehydrated in a graded ethanol series and embedded in Epon or Spurr's resin. Ultrathin sections (50 nm) were cut with a diamond knife, mounted on nickel grids, and examined.

For immunogold labeling, gills were embedded in London Resin white resin. Grids with ultrathin sections were preincubated successively in *1*) PBS-glycine (1%), *2*) PBS-gelatin (1%), and *3*) PBS-BSA (1%), each step lasting 15 min at room temperature. The grids were then incubated with MT antibody (dilution 1:200) overnight at room temperature. Control sections were incubated with BSA. After six washes in PBS-gelatin, the grids were incubated in swine anti-rabbit IgG at a dilution of 1:500 and subsequently with 10 nm colloidal gold for 1 h at room temperature. Incubated grids were then washed (3 × 5 min) in PBS and milliQ water.

Microscopy

A confocal laser scanning microscope (CLSM, MRC-600, Bio-Rad) was employed to study the colocalization of MT and Na^+-K^+-ATPase. All images were recorded and processed with an IBM compatible computer. For electron microscopic examination, a transmission electron microscope (Jeol CX11) was used at 60 kV.

Statistics

Data are presented as means ± SE. Differences among groups were assessed by ANOVA and the appropriate followup test using Instat software. Significance was accepted at 5% level.

RESULTS

Cu Concentrations in Gills and Plasma

In control fish, gill total Cu content was constant and low (around 49.7 ± 8.9 nmol/g dry wt, $n = 40$) during the 80-day period. In the Cu-exposed fish, gill Cu content increased during the first 11 days and then stabilized to a value of 786.5 ± 35.1 nmol/g dry wt ($n = 24$; pooled data from *days 14, 28, 40,* and *80*; Fig. 1). Plasma Cu concentration (12.0 ± 1.1 μmol/g) was significantly higher than in the controls (7.2 ± 0.5 μmol/g) after 80 days of Cu exposure.

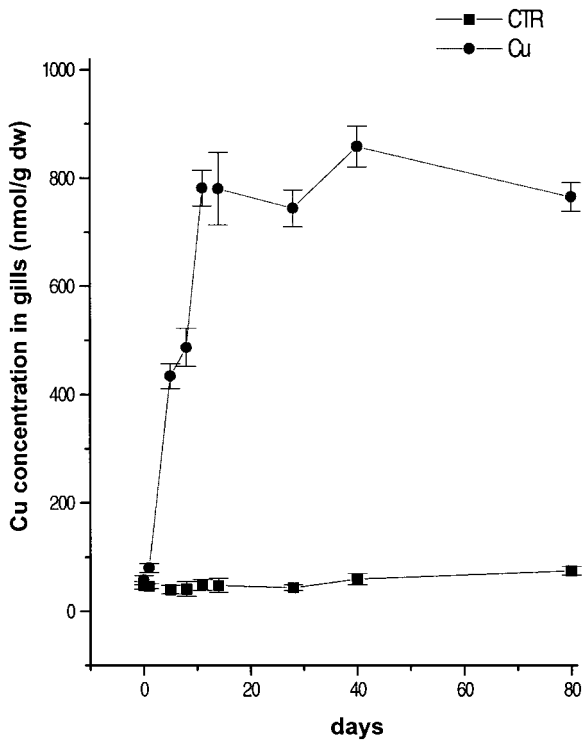

Fig. 1. Copper (Cu) concentration in tilapia gills of control (CTR) and Cu-exposed groups. Each point represents mean ± SE; $n = 6$. Significant differences ($P < 0.001$) between control and exposed fish were found after 5 days of Cu exposure.

Localization of Cu in Fish Gills

In control fish, only a weak reaction was observed in some cells in the interlamellar areas of the gill filaments (Fig. 2*A*). In Cu-exposed fish, the number of Cu-positive cells increased significantly compared with controls, with moderate to strong silver staining both in the filamental and lamellar epithelial cells (Fig. 2, *B* and *C*). In the lamellae, silver grains were limited to RC (Fig. 2*C*). More reactivity was found in the filamental than in the lamellar epithelium, and silver grains were found throughout the cytoplasm and the nuclei of these cells. Also in the basal layer cells (BLC) of the filament epithelium staining was observed (Fig. 2*D*). Neither in the filament nor in the lamellae was the staining distributed uniformly over the epithelia; it was mainly visible in sections through the trailing edge and middle part of the filaments.

A combination of AB and PAS staining for MC revealed three types of MC: AB-positive, PAS-positive, and AB- plus PAS-positive MC. Autometallography in combination with AB staining for MC in Cu-exposed fish showed that silver staining deposits were present in AB-positive as well as AB-negative MC, although not in all of these cells (Fig. 3*A*). In control fish the silver stain was found in very few MC (both AB-positive and AB-negative cells) and at low intensity. Combining PAS staining with autometallography proved to be impossible because the silver grains were removed during the oxidative step of the PAS staining. However, by using adjacent sections for the individual staining procedures, we could demonstrate that some PAS-positive MC contained Cu (results not shown).

In Cu-exposed but not in control fish, cells with the structure of CC showed Cu deposits (Fig. 3*A*). These cells were in contact with the water over a longer apical area than the MC and frequently exhibited apical pits. Furthermore, autometallography in combination with anti-Na^+-K^+-ATPase immunostaining showed colocalization of silver spots and Na^+-K^+-ATPase in such cells (Fig. 3*B*), although silver staining was sometimes obscured by DAB staining.

Localization of MT in Branchial Epithelium

Dynamics of MT induction in the gills. In gills of controls very few MT-positive cells were found; MT-positive cells were restricted to the central layer of the filamental epithelium, where most PCNA immunoreactivity was also observed (Fig. 4). Occasionally clusters of two to three MT-positive cells were seen (Fig. 5*A*). After 2 days of Cu exposure, the MT-positive cells were more numerous and bigger cells were seen than in the controls, with more variation in staining density and with a wider distribution through the epithelium. Most

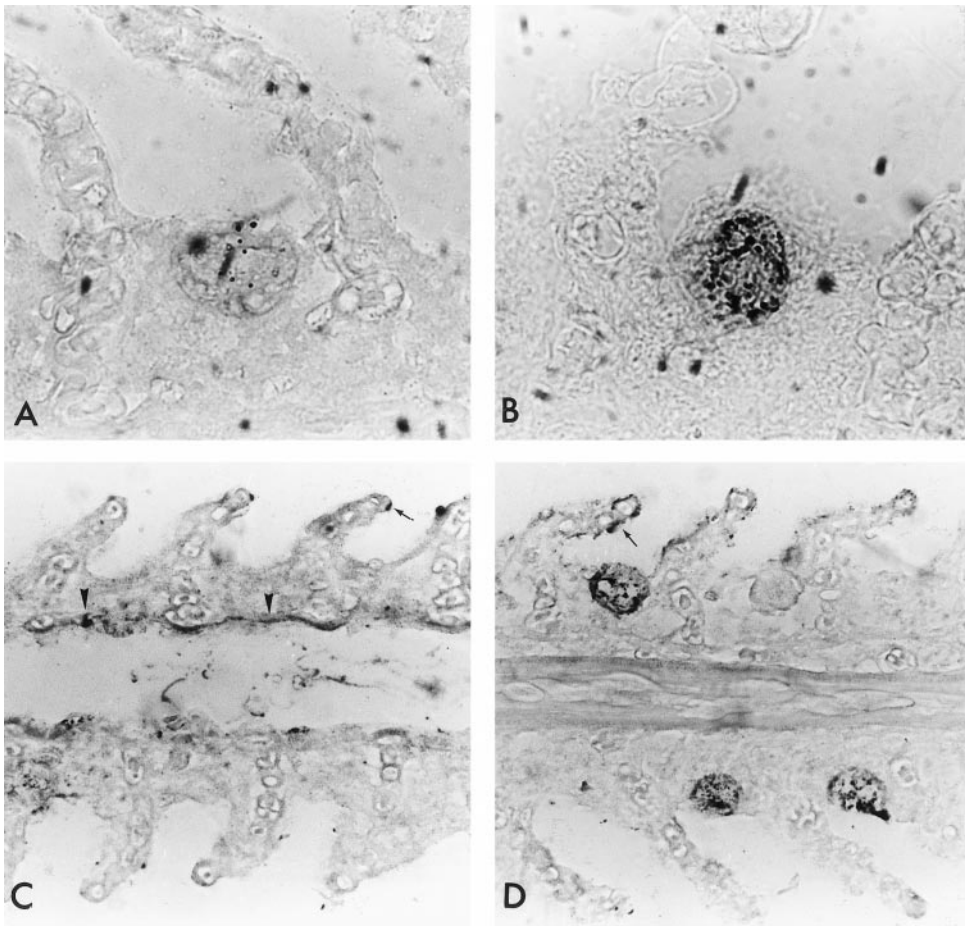

Fig. 2. Paraffin sections of gills in control and Cu-exposed tilapia. Autometallographic demonstration of Cu in gills of tilapia; a few silver grains were observed in isolated cells of the filament in control fish (*A*). After 14 days of Cu exposure, more silver grains were found in a single cell in the filament (*B*). Magnification ×630. Basal layer cells (BLC, arrowheads) in the filament (*C*) and respiratory cells (RC, arrows) in the lamellae (*C* and *D*). Magnification ×320. More Cu-positive cells were observed in fish exposed for 14 days to Cu (*D*).

were located in the upper part of the filament, in contact with the water (Fig. 5*B*). After 5 days of Cu exposure, significantly more MT-positive cells of large size and strong staining intensity could be observed (Fig. 5*C*). At the same time, in about 40% of the gill filaments MT-positive BLC were seen. After 11 days of Cu exposure, the number and density of MT-positive CC in the filaments had further increased; some MT-positive cells in the lamellae were found; MT-positive BLC were seen in 90% of the filaments (Fig. 5*D*).

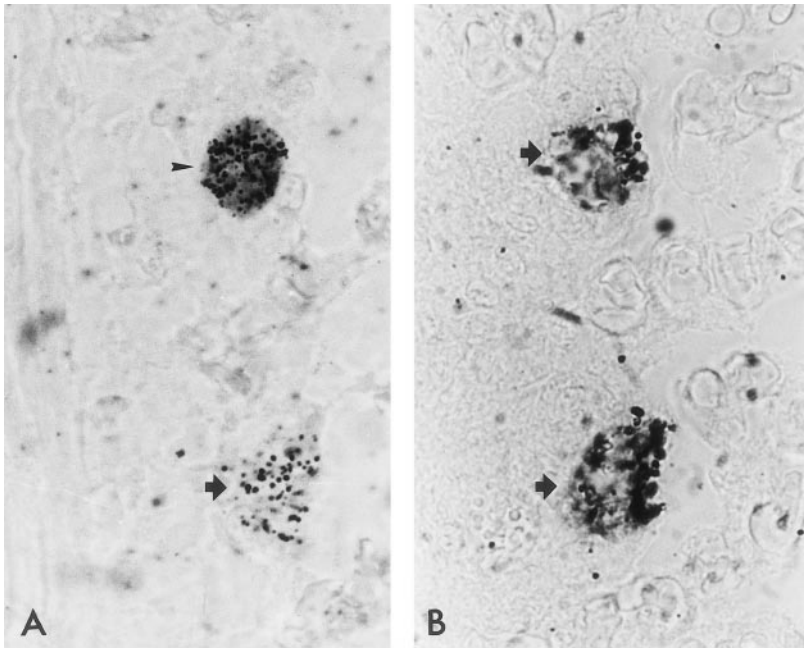

Fig. 3. Autometallography combined with alcian blue for mucous cells (MC, arrowhead) showing that MC accumulate Cu; the other cell type, with silver grains only, concerns chloride cells (CC, arrow, *A*). Autometallography and Na^+-K^+-ATPase immunostaining for CC revealed that CC (arrows) in the filament had silver grains (*B*). Magnification ×630

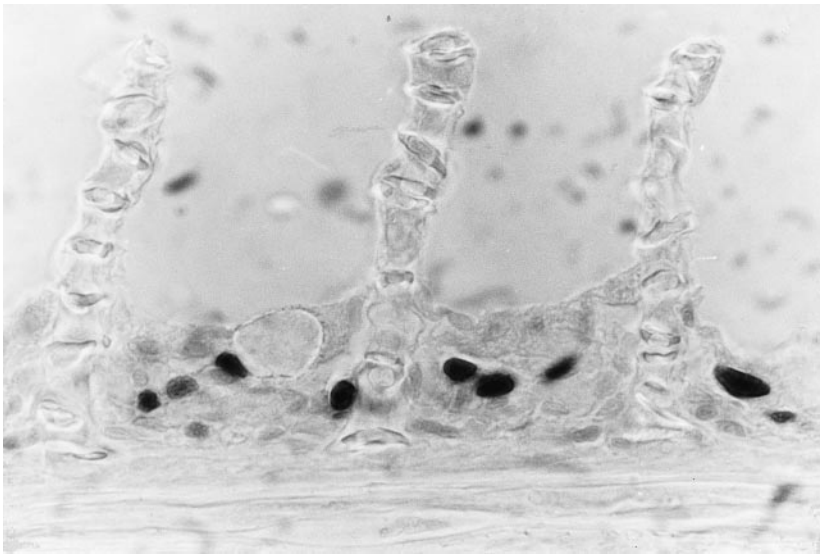

Fig. 4. Proliferating cell nuclear antigen (PCNA) staining in tilapia gills of control fish. PCNA-positive cells were mainly observed in center area of gill filament. Magnification ×630

Thereafter (*days 14*, *19*, *28*, *40*, and *80*), the MT induction plateaued.

Identification of MT-positive cell types. Confocal laser scanning microscopy of gills immunolabeled for Na^+-K^+-ATPase (Fig. 6*A*) or MT (Fig. 6*B*) or double-labeled for both (Fig. 6*C*) showed that most but not all CC (i.e., the Na^+-K^+-ATPase-positive cells) in the filament and lamellar epithelium of Cu-exposed fish contained MT. Ultrastructural examination of sections of DAB immunostained whole mounts of gill filaments (Fig. 7) gave a similar, yet more detailed picture; MT staining was found in many but not all PC of the interlamellar epithelium (Fig. 7*A*), in many RC covering the lamellae (Fig. 7*B*), in the CC, and in an occasional macrophage present in the filaments or lamellae (Fig. 7*C*). With use of immunogold labeling, MT could be demonstrated in CC (Fig. 7*D*), where the gold particles were found in all cell compartments (cytoplasm, nucleus, and mitochondria), with a preference for the apical cytoplasm. Gold labeling was also observed in PC, RC, and BLC (results not shown).

Distribution of MT-positive cell types. To localize the MT-positive cells, longitudinal as well as cross-sections of the filaments were studied. In control fish, some MT-positive cells were observed that were mainly located in the middle of the interlamellar area. After 2 days of Cu exposure, in addition to these cells, MT-positive cells appeared in the apical and basal areas of the filament epithelium. After 5 days of Cu exposure, in some of the lamellae MT-positive cells appeared. In control and 2-day Cu exposure fish MT-positive cells were exclusively found at the trailing edge of the filaments, as was best seen in cross-sections (Fig. 8*A*). After 5 days of Cu exposure, MT-positive cells were located mainly in the middle and at the trailing edge of the filaments (Fig. 8*B*). The Na^+-K^+-ATPase staining indicated that the CC were abundant in the trailing edge and the middle area of the filament, but some were also seen in the leading edge (Fig 8*C*). In this latter area no MT-positive cells were found, indicating that the CC in this area did not express MT. After 5 days of Cu exposure, MT-positive BLC were observed in the trailing edge and the middle area of the filament (Fig. 8*B*). This distribution was reflected by prominent staining of basal cells in longitudinal sections through the middle of the filament (Fig. 9*A*) and by lamellar staining in filament sections more closely to the leading edge (Fig. 9*B*). In longitudinal sections positive PC, CC, and BLC were observed, more at the distal parts and at the base of the filaments than in the middle parts.

DISCUSSION

Our results are the first to demonstrate the cellular location of Cu and MT in gills of Cu-exposed fish. Both Cu and MT were found in CC, PC, RC, and BLC of gill epithelium after exposure to Cu in the water. Cu, but not MT, was observed in MC. The analysis of the time course and location of MT induction in the gills of Cu-exposed fish indicated that the metal binding proteins did not come to expression in fully differentiated cells but followed the route of newly recruited branchial cells as illustrated in Fig. 10.

Cu accumulated rapidly in the gills and plateaued after 11 days, in line with earlier observations on Cu-exposed tilapia (23, 30). The autometallography procedure used in this study to detect Cu is a technique with low metal specificity, and thus the positive staining may reflect the presence of a variety of heavy metals such as Cu, Zn, or Cd that are present as traces in tap water and the food used in our experiments. However, after exposure to 3.2 μmol/l waterborne Cu, we observed a marked increase in the intensity of silver staining in the different branchial cell types, and this most likely results from the accumulation of Cu in these cells. In the Cu-exposed fish, the distribution of Cu in the branchial epithelium was not homogeneous, with more Cu being found in the filaments than in the lamellae and more silver grains per filament cell.

Cu could be localized in every cell type of the branchial epithelium, including CC, PC, RC, and BLC, as well as MC. However, it appeared that only a small

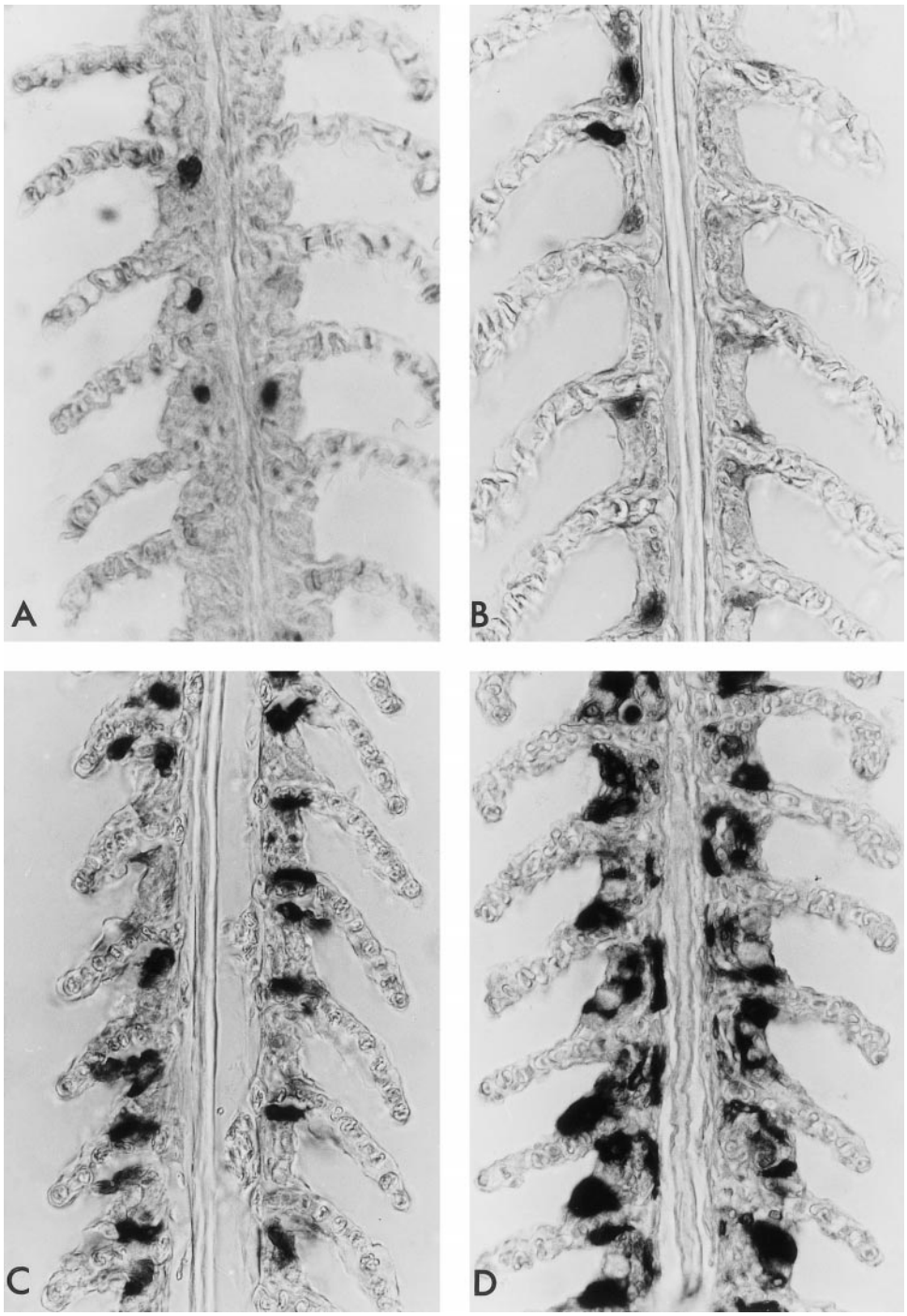

Fig. 5. Dynamics of metallothionein (MT) in tilapia gills after Cu exposure. MT in control tilapia gills were restricted to center layer of filamental epithelium. Occasionally 2–3 MT-positive cells were found in this area (*A*). After 2 days of Cu exposure, MT-positive cells in filament were more numerous and bigger, with more variation in staining density (*B*). After 5 days of Cu exposure, even more MT-positive cells, with stronger staining density were found in the filament (*C*). After 11 days of Cu exposure, MT-positive cell numbers increased further (*D*). Magnification ×320

fraction of the cells was stained and with a varying staining intensity. We have found the Na^+-K^+-ATPase immunoreactivity as marker for the CC population and this proved to be valid as our (unpublished) ultrastructural observations indicate that Na^+-K^+-ATPase immunoreactivity is restricted to these cells in tilapia. The presence of Cu in CC was demonstrated by autometallography together with immunostaining for Na^+-K^+-ATPase for CC (DAB procedure) on the same section or on adjacent sections. A combination of AB staining and autometallography allowed the MC to be identified as Cu-accumulating cells. Not all AB-positive cells contained Cu, but this may reflect differences in the developmental stage of AB-positive cells. Moreover, not all MC were AB positive as was indicated by the presence of cells with neutral (AB negative but PAS positive) mucus products. Similar categories of MC have been described for brown trout (1). Cu binding occurred in both AB-positive and AB-negative MC and thus appears to be independent of the kind of mucus produced (i.e., acid or neutral components).

As Cu is sequestered by MT intracellularly (14), Cu staining may be predicted to reflect the distribution of MT. Indeed, similar staining patterns for Cu and MT

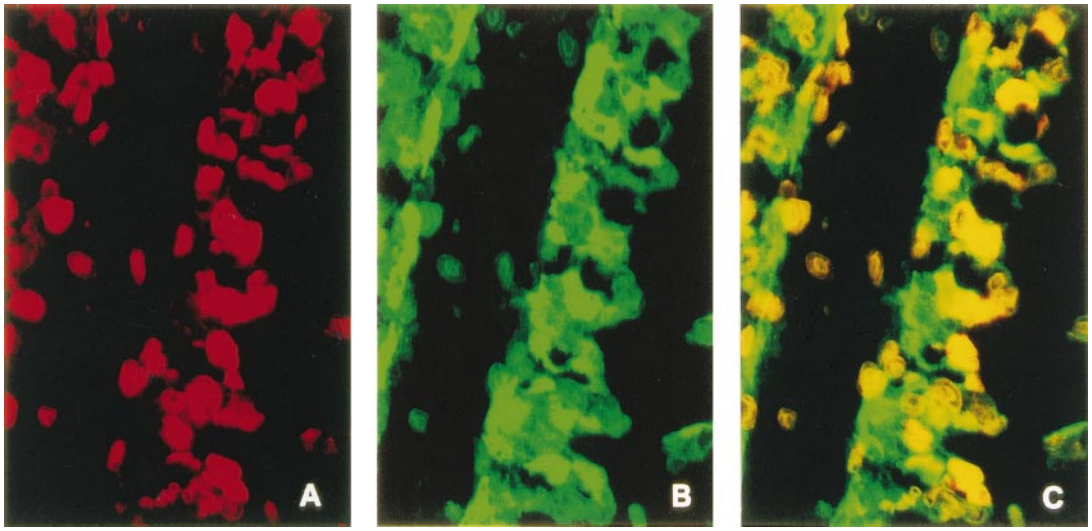

Fig. 6. Confocal image of gill section doubly stained for Na^+-K^+-ATPase and MT. *A*: Na^+-K^+-ATPase antigenicity. *B*: MT antigenicity. *C*: confocal images *A* and *B* were merged to demonstrate partial colocalization of MT and Na^+-K^+-ATPase immunopositive sites. Magnification ×400

were found in PC, RC, and CC, as well as in BLC and some macrophages. Thus our data clearly demonstrate that Cu can induce MT in fish gills. This induction runs parallel with the increase of Cu concentration in gills of fish exposed to Cu in water; after the first signs of MT induction on *day 2*, the number and size of MT-positive cells had increased rapidly at *day 5* and had reached a maximum at *day 11*, similar to the pattern for Cu accumulation. The presence of MT in these cells was based on light and electron microscope (LM and EM) observations with a combination of techniques. In all cell types it was found that both cytoplasm and nucleus were stained. Our conclusion that the CC were MT positive is based on two observations. First, at the LM level with the CLSM we showed that double staining of gills for Na^+-K^+-ATPase and MT revealed significant overlap. However, some MT-negative, Na^+-K^+-ATPase immunoreactive (ir)-positive cells were found. Second, at the EM level, MT immunogold labeling was found in the CC. Within the CC, MT was distributed asymmetrically, more being present in the apical part of the cells, perhaps to form a protective barrier for the delicate Na^+-K^+-ATPase activity, which has a high sensitivity to Cu and is located in the tubular system in the middle and basal areas of these cells (22).

The MT-positive CC that appeared during the first days of Cu exposure were probably newly differentiated cells. We base this conclusion on the following observations. First, whereas no MT-positive CC were observed in control fish, the first MT-positive cells appeared in the center of the filament epithelium, where cells express PCNA, the area where probably all branchial epithelial cells originate (18, 33). Second, MT did not appear in the fully differentiated CC before *day 2*. It is known that the differentiation of CC takes about 4 days in guppy (5) and a few days in chum salmon (37). The fact that MT staining intensity in CC is variable after Cu exposure indicates that different developmental stages of CC contain different amounts of MT. Third, the time course and location of MT induction indicate that MT-positive cells migrate in about 2 to 5 days from the middle area to the upper layers of the filament epithelium (CC, PC), down to the basal layers (BLC) and to the lamellar RC (Fig. 10). The MT-positive PC are probably also newly differentiated cells because it takes several days of Cu exposure before MT-positive PC could be detected. This also holds for RC, where MT positivity also developed slowly. Apparently, in mature and pre-existing PC and RC Cu cannot induce expression of MT. Similar to the CC, these cells probably have to be replaced by new cells before a protective action of MT can become effective.

Fish gill filaments are asymmetrical and complex in structure (18, 40). Comparing the distribution of CC and MT-positive cells, we found the latter concentrated in the trailing edge of the filament. In control fish, MT-positive cells were very scarce and distributed irregularly in the central layer of the filamental epithelium. In this area most of the undifferentiated cells are known to be present (19, 33), and it is also here that mitosis can be demonstrated in the tilapia gill, with the PCNA technique in this study as well as by electron microscopic examination (our unpublished observations). This is also in line with observations on guppy and rainbow trout employing [^{3}H]thymidine radioautography techniques (5) and 5-bromo-2′-deoxyuridine techniques (19), respectively. However, in this area also most macrophages occur in control fish, and since we observed that these were MT positive it cannot be excluded that the occasional MT-positive cells in the controls represent macrophages rather than undifferentiated cells. The MT induction in Cu-exposed fish became first and predominantly visible in the middle and trailing edges of the filaments. Induction of MT-positive cells did not occur simultaneously nor homogeneously in all filaments and in different areas of the filaments. So the picture arises that cells expressing MT in their mature state, and this applied to all cell

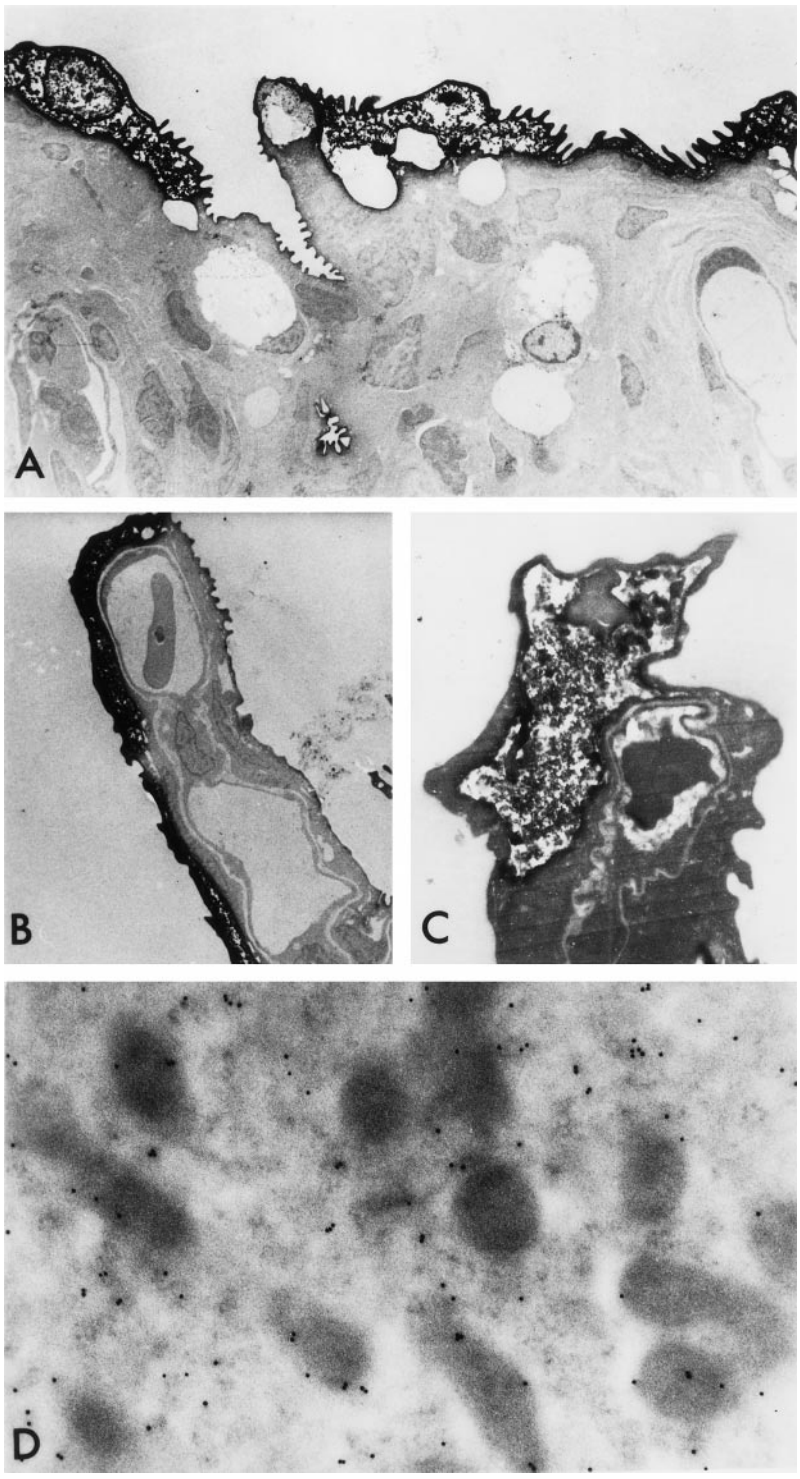

Fig. 7. Ultrastructure of gills stained by 3,3′-diaminobenzidine, which show that pavement cells (PC) (*A*), RC (*B*), and macrophages (*C*) are MT positive. Magnification ×3,000. Immunogold labeling showed that gold particles appeared in CC (*D*). Magnification ×10,000.

types of the branchial epithelium, must have been exposed to Cu during an earlier phase of their cell cycle; the observation that CC in the trailing edge first become irMT positive would suggest a higher turnover of these cells compared with PC and RC. We conclude from our results that MT induction occurs first in the trailing edge of the filament, i.e., those areas where the cells appear to be exposed to the highest concentrations of Cu. Indeed, this is the area with the highest numbers of CC and ion transport activity (18, 31).

High Cu accumulation in fish gills is usually associated with tissue damage and disturbed branchial functioning (25, 26). A prime action of Cu in fish is the disturbance of Na^+ transport in the gills (15, 30). The copper ion (Cu^{2+}) has a very high affinity for cysteine groups (16, 34) and therefore may exert toxic effects by binding to cysteine-rich proteins such as Na^+-K^+-ATPase (22). This might explain both the high incidence of necrotic and apoptotic cells as well as inhibition of Na^+ transport in Cu-exposed fish (22, 30).

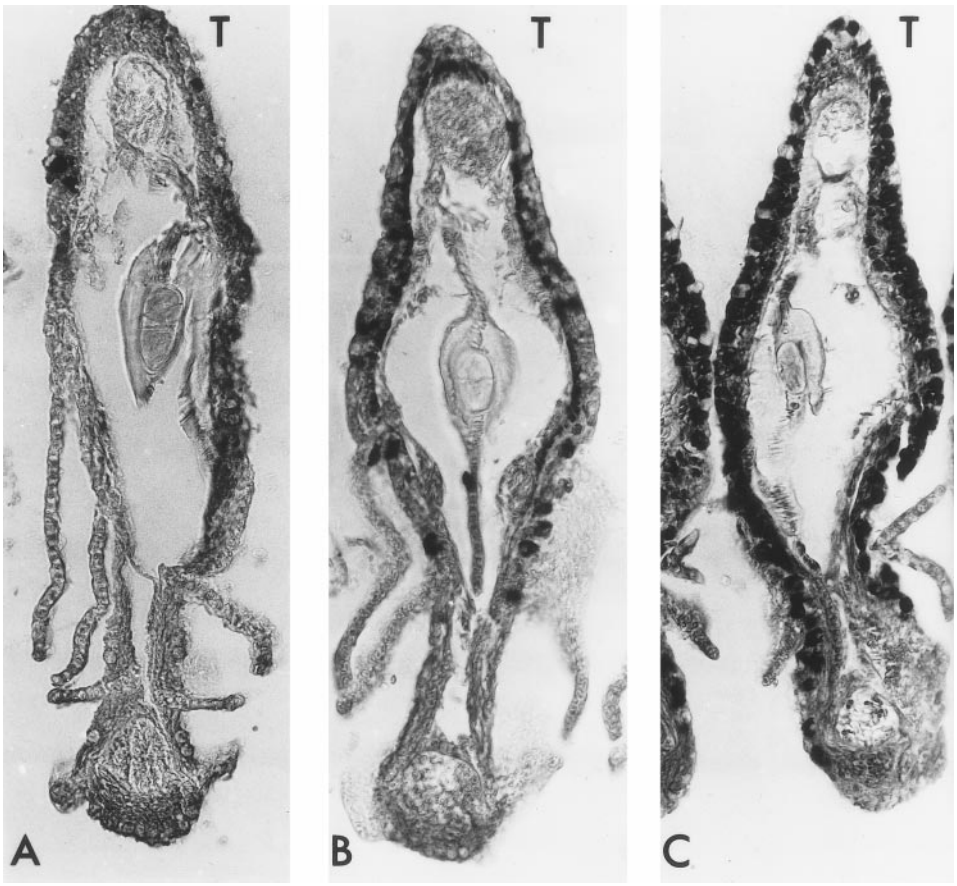

Fig. 8. MT-positive cell distribution over trailing edge (T) of filament. *A*: 2-day Cu-exposed fish. *B*: 14-day Cu-exposed fish. *C*: Na^+-K^+-ATPase immunostaining for CC. Magnification ×160.

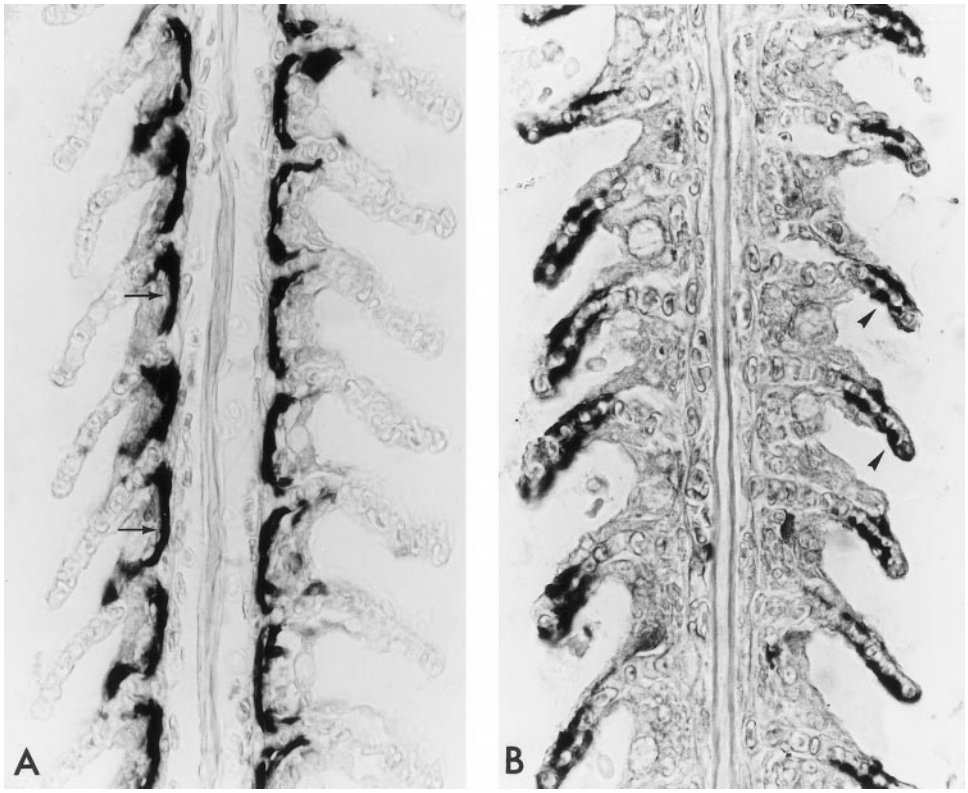

Fig. 9. MT immunostaining after 14 days of Cu exposure, with BLC (arrows) in the filament (*A*) and RC (arrowheads) in the lamellae (*B*). Magnification ×320.

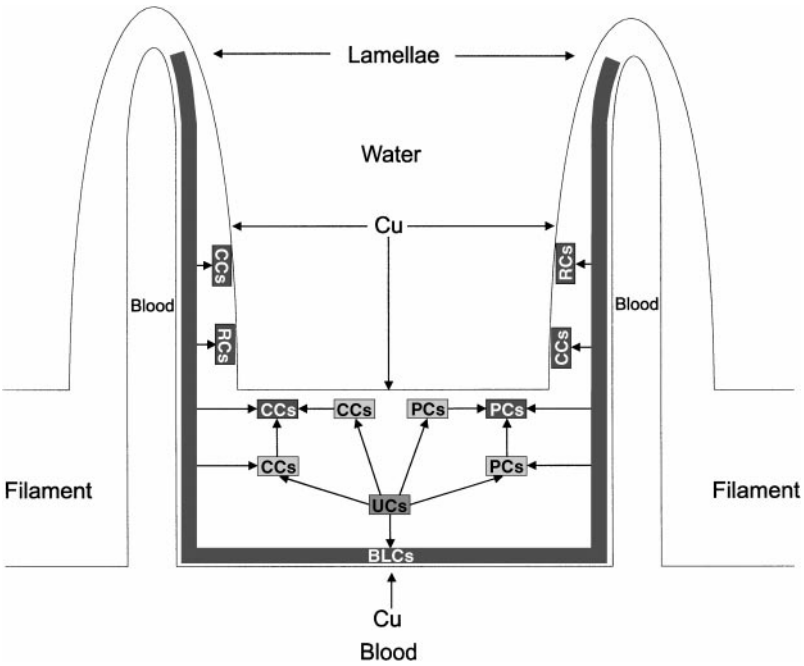

Fig. 10. Model for MT induction in tilapia gills after exposure to waterborne Cu. Gray shading intensity indicates amount of MT in cells. After Cu exposure, undifferentiated cells (UCs) in PCNA expression area can develop into CC or PC; with progressing differentiation MT expression increases. After prolonged exposure to Cu, BLC form a sheet of MT-positive cells. This sheet is located predominately in those areas of the gills where active CC occur and where transport of ions, including Cu^{2+}, occurs.

On the assumption that newly differentiated irMT-positive CC are protected against Cu toxicity and irMT-positive PC represent a protective barrier in gills, MT may contribute to the restoration of transport of Na^+ and other ions in the gills. This would also explain the reduction of apoptosis and necrosis of the CC after the first week of Cu exposure (23). The reduction in epithelial lifting (27) and the restoration of plasma sodium levels (17) seen on the longer term in rainbow trout exposed to Cu were also observed in our tilapia (data not shown), and such observations indicate a successful adaptative response to which the MT-induction may contribute significantly.

Our EM and LM results demonstrate that MT was present both in the cytoplasm and nuclei of CC, PC, and RC, although it was unevenly distributed over the cytoplasm. It is well known for mammalian cells that irMT can be demonstrated in all cell compartments, including the nucleus (3, 4, 21). Nuclear MT may serve a protective role against the genotoxic effects of heavy metals (39). The apparent translocation of MT synthesized in the cytosol into the nucleus could serve a dual protective function, either the binding of heavy metals or the scavenging of free radicals (3).

Cu induces necrosis and apoptosis of PC, RC, as well as CC (23, 30). This may be a result of the accumulation of Cu and damage to the cells by the metal, and thus Cu exposure may accelerate replacement of cells (30). The involvement of macrophages in the removal and digestion of apoptotic bodies of the CC (41) may explain why these cells also show MT staining. This presence of MT in macrophages has not been demonstrated before for fish but is in line with observations on mammalian macrophages (7, 11, 20). Thus Cu will be removed by sloughing-off of cells into the water (PC, RC, and MC) or by macrophages, which are known to remove apoptotic CC by phagocytosis (41). These processes may determine the stabilization of the metal content of the gills after 11 days.

After 5 days of Cu exposure, more and more cells from the basal layer of the filament epithelium became irMT positive. These BLC may serve two functions, i.e., acting as the progenitor cells of CC, PC, and RC (18) and forming a protective barrier at the base of the branchial epithelium. Our observation that PCNA expression occurs in cells positioned centrally in the filamental epithelium would favor the interpretation that BLC are differentiated to function as a protective barrier. After Cu exposure, the blood Cu concentration increases significantly (30). Therefore, the gill epithelium could be affected by Cu entering from the water as well as from the blood. As BLC are bordering on the blood compartment, these cells are likely to be affected more by Cu from the blood than from the water. Even after 80 days of Cu exposure, we observed that the Cu concentration in the blood was still higher than in controls, and coincidentally BLC were found to be MT positive. The staining intensity of this cell layer was strong, which indicates that MT content in these cells was high.

Our results demonstrate that at least part of the AB-positive MC accumulated Cu as shown by autometallography. Nevertheless, all MC were negative for MT staining, shown at both LM and EM level. This indicates that not only MT but also mucous compounds can bind Cu. In particular glycoproteins and proteoglycans present in mucus have a strong affinity for heavy metals, including Cu (38). Mucus covering the gills may therefore also represent a first line of defense against Cu exposure. Elevated branchial mucous concentrations in fish during waterborne metal exposure have often been suggested to serve a detoxifying role (10, 35). The increased release of mucus with Cu binding capac-

ity may contribute to explain why total Cu content of the gills stabilizes after 11 days of Cu exposure in addition to cell removal.

Perspectives

Our results indicate that there is a window for Cu-inducible MT expression in the early stages of cell development and once induced, MT remains expressed for the rest of the life cycle of these cells. The model we propose shows a parallel between the time course of Cu-induced MT staining in the gill and of the epithelial cell development. Thus irMT expression on Cu exposure is a marker for the cell development in tilapia gills, which may further elucidate cell recruitment during adaptation to stress conditions. Whether this model may also be applied to other species of fish is presently under investigation in our laboratory. For a long time, the function of BLC in the gill filament has been unclear, but our results strongly favor the interpretations that these cells may form a protective barrier in the filamental epithelium for heavy metal ions and differentiate into the functional CC, PC, and RC.

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REFERENCES

1. **Blackstock, N., and A. D. Pickering.** Changes in the concentration and histochemistry of epidermal mucous cells during the alevin and fry stages of the brown trout *Salmo trutta*. *J. Zool. Lond*. 197: 463–471, 1982.
2. **Bury, N. R., J. Li, G. Flik, R. A. C. Lock, and S. E. Wendelaar Bonga.** Cortisol protects against copper induced necrosis and promotes apoptosis in fish gill chloride cells in vitro. *Aquat. Toxicol. (Amst.)* 40: 193–202, 1998.
3. **Cherian, M. G.** The significance of the nuclear and cytoplasmic localization of metallothionein in human liver and tumor cells. *Environ. Health Perspect*. 02, *Suppl*. 3: 131–135, 1994.
4. **Cherian, M. G., and M. Nordberg.** Cellular adaptation in metal toxicology and metallothionein. *Toxicology* 28: 1–15, 1983.
5. **Chretien, M., and M. Pisam.** Cell renewal and differentiation in the gill epithelium of fresh- or salt-water-adapted euryhaline fish as revealed by [^{3}H]thymidine radioautography. *Biol. Cell* 56: 137–150, 1986.
6. **Danscher, G.** Autometallography—a new technique for light and electron microscopic visualization of metals in biological tissues (gold, silver, metal sulphides and metal selenides). *Histochemistry* 81: 331–335, 1984.
7. **Douglas-Jones, A. G., N. D. Thomas, M. E. Elmes, and B. Jasani.** Immunocytochemically detectable metallothionein in granulation tissue surrounding mucosal ulceration. *Histochem. J*. 24: 40–50, 1992.
8. **Dutta, H. M., J. S. D. Munshi, P. K. Roy, N. K. Singh, S. Adhikari, and J. Killius.** Ultrastructural changes in the respiratory lamellae of the catfish, *Heteropneustes fossilis* after sublethal exposure to malathion. *Environ. Pollut*. 92: 329–341, 1996.
9. **Fu, H., O. M. Steinebach, C. J. A. van den Hamer, P. H. M. Balm, and R. A. C. Lock.** Involvement of cortisol and metallothionein-like proteins in the physiological responses of tilapia (*Oreochromis mossambicus*) to sublethal cadmium stress. *Aquat. Toxicol. (Amst.)* 16: 257–270, 1990.
10. **Handy, R. D., and F. B. Eddy.** The interactions between the surface of rainbow trout, *Oncorhynchus mykiss*, and waterborne metal toxicants. *Funct. Ecol*. 4: 385–392, 1990.
11. **Hart, B. A., Q. Gong, J. D. Eneman, C. C. Durieux-Lu, P. Kimberly, and M. P. Hacker.** Increased oxidant resistance of alveolar macrophages isolated from rats repeatedly exposed to cadmium aerosols. *Toxicology* 107: 163–175, 1996.
12. **Hogstrand, C., F. Galvez, and C. M. Wood.** Toxicity, silver accumulation and metallothionein induction in freshwater rainbow trout during exposure to different silver salts. *Environ. Toxicol. Chem*. 15: 1102–1108, 1996.
13. **Hsu, S. M., L. Raine, and H. Fanger.** Use of avidin-biotin-peroxidase complex (ABC) and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem*. 29: 577–580, 1981.
14. **Kägi, J. H. R.** Evolution, structure and chemical activity of class I metallothioneins: an overview. In: *Metallothioneins III: Biological Roles and Medical Implications*, edited by K. T. Suzuki, N. Imura, and M. B. Kimura. Basel: Verlag, 1993, p. 29–55.
15. **Laurén, D. J., and D. G. McDonald.** Effects of copper on branchial ionoregulation in the rainbow trout, *Salmo gairdneri* Richardson. *J. Comp. Physiol. [B]* 155: 635–644, 1985.
16. **Laurén, D. J., and D. G. McDonald.** Influence of water hardness, pH, and alkalinity on the mechanisms of copper toxicity in juvenile rainbow trout, *Salmo gairdneri*. *Can. J. Fish. Aquat. Sci*. 43: 1488–1496, 1986.
17. **Laurén, D. J., and D. G. McDonald.** Acclimation to copper by rainbow trout, *Salmo gairdneri*: physiology. *Can. J. Fish. Aquat. Sci*. 44: 99–104, 1987.
18. **Laurent, P.** Gill internal morphology. In: *Fish Physiology*, edited by W. S. Hoar and D. J. Randall. New York: Academic, 1984, vol. XA, p. 73–183.
19. **Laurent, P., S. Dunel-Erb, C. Chevalier, and J. Lignon.** Gill epithelial cells kinetics in a freshwater teleost, *Oncorhynchus mykiss* during adaptation to ion-poor water and hormonal treatments. *Fish Physiol. Biochem*. 13: 353–370, 1994.
20. **Laurin, D. E., and K. C. Klasing.** Role of synthesis and degradation in the regulation of metallothionein accretion in a chicken macrophage-cell line. *Biochem. J*. 268: 459–463, 1990.
21. **Leyshon-Sorland, K., and E. Strang.** The ultrastructural localization of metallothionein in cadmium exposed rat liver. *Histochem. J*. 25: 857–864, 1993.
22. **Li, J., R. A. C. Lock, P. H. M. Klaren, H. G. P. Swarts, F. M. A. H. Schuurmans Stekhoven, S. E. Wendelaar Bonga, and G. Flik.** Kinetics of Cu inhibition of Na^+-K^+-ATPase. *Toxicol. Lett*. 87: 31–38, 1996.
23. **Li, J., E. S. Quabius, S. E. Wendelaar Bonga, G. Flik, and R. A. C. Lock.** Effects of waterborne copper on branchial chloride cells and Na^+/V^+-ATPase activities in Mozambique tilapia (*Oreochromis mossambicus*). *Aquat. Toxicol. (Amst.)* 43:1–10, 1998.
24. **Lin, H., and D. Randall.** Proton pumps in fish gills. In: *Cellular and Molecular Approaches to Fish Ionic Regulation*, edited by C. M. Wood and T. J. Shuttleworth. San Diego, CA: Academic, 1995, p. 229–256.
25. **Mallatt, J.** Fish gill structure changes induced by toxicants and other irritants: a statistical review. *Can. J. Fish. Aquat. Sci*. 42: 630–648, 1985.
26. **Marr, J. C. A., H. L. Bergman, J. Lipton, and C. Hogstrand.** Differences in relative sensitivity of naive and metals-acclimated brown and rainbow trout exposed to metals representative of the Clark Fork River, Montana. *Can. J. Fish. Aquat. Sci*. 52: 2016–2030, 1995.
27. **McDonald, D. G., and C. M. Wood.** Branchial mechanisms of acclimation to metals in freshwater fish. In: *Fish Ecophysiology*, edited by J. C. Rankin, and F. B. Jensen. London, UK: Chapman and Hall, 1993, p. 297–332.
28. **Norey, C. G., A. Cryer, and J. Kay.** A comparison of cadmium-induced metallothionein gene expression and metal ion distribution in the tissues of cadmium-sensitive (rainbow trout, *Salmo gairdneri*) and tolerant (stone loach, *Noemacheilus barbatulus*) species of freshwater fish. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol*. 97: 221–226, 1990.
29. **Olsson, P. E.** Metallothioneins in fish: induction and use in environmental monitoring. In: *Toxicology of Aquatic Pollution:*

Physiological, Cellular and Molecular Approaches, edited by E. W. Taylor. Cambridge, UK: Cambridge University Press, 1996, p. 187–203.

30. **Pelgrom, S. M. G. J., R. A. C. Lock, P. H. M. Balm, and S. E. Wendelaar Bonga.** Integrated physiological responses of tilapia, *Oreochromis mossambicus*, to sublethal copper exposure and endocrine regulation. *Aquat. Toxicol. (Amst.)* 32: 303–320, 1995.
31. **Perry, S. F.** The chloride cell: structure and function in the gills of freshwater fishes. *Annu. Rev. Physiol*. 59: 325–347, 1997.
32. **Perry, S. F., and P. Laurent.** Environmental effects on fish gill structure and function. In: *Fish Ecophysiology*, edited by J. C. Rankin and F. B. Jensen. London, UK: Chapman & Hall, 1993, p. 233–263.
33. **Pisam, M., and A. Rambourg.** Mitochondria-rich cells in the gill epithelium of teleost fishes: an ultrastructural approach. *Int. Rev. Cytol*. 130: 191–232, 1991.
34. **Reid, S. D., and D. G. McDonald.** Metal binding activity of the gills of rainbow trout (*Oncorhynchus mykiss*). *Can. J. Fish. Aquat. Sci*. 48: 1061–1068, 1991.
35. **Shephard, K. L.** Functions for fish mucus. *Rev. Fish. Biol. Fisher*. 4: 401–429, 1994.
36. **Soto, M., M. P. Cajaraville, E. Angulo, and I. Marigomez.** Autometallographic localization of protein-bound copper and zinc in the common winkle, *Littorina littorea*: a light microscopical study. *Histochem. J*. 28: 689–701, 1996.
37. **Uchida, K., and T. Kaneko.** Enhanced chloride cell turnover in the gills of chum salmon fry in seawater. *Zool. Sci*. 13: 655–660, 1996.
38. **Van de Winkel, J. G. J., T. H. M. S. van Kuppevelt, H. M. J. Janssen, and R. A. C. Lock.** Glycosaminoglycans in the skin mucus of rainbow trout (*Salmo gairdneri*). *Comp. Biochem. Physiol. B Biochem. Mol. Biol*. 85: 473–475, 1986.
39. **Waalkes, M. P., T. P. Coogan, and R. A. Barter.** Toxicological principles of metal carcinogenesis with special emphasis on cadmium. *Crit. Rev. Toxicol*. 22: 175–201, 1992.
40. **Wendelaar Bonga, S. E.** The stress response in fish. *Physiol. Rev*. 77: 591–625, 1997.
41. **Wendelaar Bonga, S. E., G. Flik, P. H. M. Balm, and J. C. A. van der Meij.** The ultrastructure of chloride cells in the gills of the teleost *Oreochromis mossambicus* during exposure to acidified water. *Cell Tissue Res*. 259: 575–585, 1990.