Histol Histopathol (2003) 18: 1095-1101

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

# Distribution of DMT 1 within the human glandular system

**R.O. Koch[2], H. Zoller[1,2], I. Theurl[1], P. Obrist[3], G. Egg[2], W. Strohmayer[4], W. Vogel[2] and G. Weiss[1]**

[1]Department of Internal Medicine and [2]Division of Gastroenterology and Hepatology, University Hospital of Innsbruck, Austria, [3]Department of Pathology, University of Innsbruck, Austria and [4]Institute for Experimental Traumatology, Vienna, Austria

**Summary.** Background & Aims: DMT1 is a transmembrane protein which transports the divalent metal ions $Fe^{2+}$, $Zn^{2+}$, $Cu^{2+}$ and $Mn^{2+}$. Although DMT1 has been functionally linked to duodenal absorption and cellular utilisation of iron hardly anything is known about its distribution and potential role within the human glandular system. Methods: Two polyclonal antibodies were raised to study the expression of DMT1 in tissues obtained from human corpus by the means of immunocytochemistry and Western blotting. Results: All antibodies specifically detected a 60 kD protein band referring to human DMT1. Significant amounts of DMT1 expression were detected on the luminal site of organs, which are involved in excretion/re-absorption processes, such as salivary glands, pancreas, biliary tract and gallbladder. Conclusions: Our results suggest that DMT1 may be of pivotal importance for the regulation of metal ion homeostasis within organs involved in absorption and excretion of ions.

**Key words:** DMT1, Glandulae, Hemochromatosis, Pancreas, Ion transport

## Introduction

Divalent metal transporter 1 (DMT1) is an integral membrane-bound transport protein with 12 putative transmembrane domains and several N-linked glycosylation sites (Fleming, 1997). DMT1 mRNA injections into Xenopus oocytes increased the uptake of divalent cations such as $Fe^{2+}$, $Zn^{2+}$, $Cu^{2+}$ and $Mn^{2+}$ in a pH-dependent fashion by a proton-coupled process (Gunshin, 1997). In intestinal cell lines DMT1 expression depends on iron availability, with increased expression during iron deprivation status (Zoller, 2001). The underlying mechanism for this may be referred to the presence of an iron-responsive element (IRE) within the 3´untranslated region of DMT1 mRNA (Lee 1998). In a comparable fashion to what is known for the regulation of transferrin receptor (TfR) mRNA by iron this IRE may be targeted by iron regulatory proteins (IRPs), which are activated during iron deprivation. Binding of IRPs to IRE stem loop structures results in an increased stability of TfR mRNA and presumably DMT1 mRNA by preventing its degradation by an RNAse (Gunshin 2001; Hentze and Kuhn 1996; Rouault and Klausner 1997). Nonetheless, evidence has been provided that DMT1 expression is also regulated by iron availability at the transcriptional level (Tchernitchko, 2002; Zoller, 2002). Moreover, a missense mutation of DMT1 has been shown to be associated with an impaired delivery of iron to erythroid cells and a subsequent induction of microcytic anemia (Fleming, 1997; Su, 1998). Although DMT1 has turned out to be a functionally important transmembrane transporter, hardly anything is known about quantitative expression and tissue distribution of DMT1 in tissues with excretory function or its potential physiological role in humans.

*Offprint requests to:* Günter Weiss, MD, Department of Internal Medicine, anichstrasse 35, A-6020 Innsbruck, Austria. Fax: ++43 512 504 5607. e-mail: guenter.weiss@uibk.ac.at

## Materials and methods

### *Materials*

Affinity-purified alkaline phosphatase-conjugated goat anti-rabbit IgG, nitro blue tetrazolium, 5-bromo-4-chloro-3-indoyl phosphate, Tween 20, deoxycholate, pepstatin, leupeptin and Triton X-100 were all obtained from Sigma (Diesenhofen, Germany) and streptavidin-peroxidase complex as well as biotin-coupled goat anti-rabbit IgG were purchased from DAKO (Vienna, Austria). Pre-stained molecular mass standards (range 215,000 to 7,800) and Immobilon polyvinylidene difluoride membranes were purchased from Bio-Rad (Richmond, CA). CNBr-activated Sepharose 4B was obtained from Amersham (Uppsala, Sweden). All reagents were of the highest commercially available purity grade.

### *Antibody production*

Rabbit polyclonal sera were raised against putative

extracellular loops of DMT1. The sequence of the synthetic peptides used and their location within the primary amino acid sequences were KPSQSQVLKGMTVP (anti-DMT1(174-187)) and VFAEAFFGKTNEQVVE (anti-DMT1(260-275)), according to the cDNA sequence published by Kishi (Kishi and Tabuchi, 1997). Crude serum was affinity purified. Antibody production and affinity purification were performed as previously described (Koch et al., 2001; Zoller et al., 2002).

### *Preparation of human tissue homogenate*

The human tissues investigated in these series were freshly obtained either from surgical specimens or from postmortem examinations. For Western blotting analysis tissue was lysed in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris HCl pH 8.0, 0.2 mM PMSF, 1 $\mu$g/ml pepstatin, 0.5 $\mu$g/ml leupeptin) at 4 ºC for 30 min and subsequently centrifuged at 16.000g for 5 min.

### *Western blotting*

Protein concentration was determined according to the Bradford method and 15-20 $\mu$g of protein lysates were loaded onto a 10% SDS polyacrylamide gel. Proteins were transferred under constant cooling in 25 mM Tris, 192 mM glycine and 10% (v/v) methanol from 10% polyacrylamide gel onto PVDF membranes for 60 min at a constant voltage (100V). After protein transfer, the PVDF membrane was incubated with 20mM Tris-HCl (pH 7.4) containing 10% skimmed milk powder, 0.5% (w/v) Triton X-100, and 0.1% Tween 20 and 150 mM NaCl (TBS) for 12-18 hours at 4°C. Subsequently, the immunoblot was incubated with affinity-purified antibodies directed against DMT1 at a dilution of 1:200 in the same buffer for 12 hours at 22 °C. After washing with 0.5% (w/v) Triton X-100, and 0.1% Tween 20 in TBS, blots were incubated with affinity-purified alkaline phosphatase-conjugated goat anti-rabbit IgG at a 1:1500 dilution for 60 min at 22 °C. After rinsing several times with Triton buffer, the blots were developed in 100 mM Tris-HCl (pH 9.5), 100 mM NaCl and 5 mM $MgCl_2$, 0,033% NBT and 0.017% BCIP.

### *Immunocytochemistry*

4 $\mu$m paraffin sections, which were previously fixed in 10% formaldehyd for 24 hours, were used for immunohistochemistry. Endogenous peroxidase was inactivated by treatment with methanol / 0.5% $H_2O_2$ for 20 minutes. Thereafter, sections were incubated in 0.1ml 300$\mu$g/ml affinity-purified anti-DMT1(260-275) antiserum at 4 °C for 20 hours. After washing with Tris-HCl (pH 7.4), slides were incubated with a biotin-coupled goat anti-rabbit IgG in a 1:500 dilution for 30 minutes, followed by two washes with Tris-HCl (pH 7.4). Subsequently, slides were incubated with 0.1ml of streptavidin-peroxidase complex 1:800 diluted in PBS with 1% BSA. Visualization of the antibody antigen complexes was done with 0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide. Sections for control staining were treated as detailed above, and incubated with the breakthrough from the affinity purification. For all investigations controls employing pre-immune serums or without the use of DMT-1 antibodies were performed which in no case showed specific staining or a considerable background staining.

## Results

### *Production and characterization of DMT1-specific antibodies*

Two antisera against extracellular loops of DMT1, anti-DMT1($_{174-187}$) and anti-DMT1($_{260-275}$), respectively, were generated in rabbits and affinity purified subsequently. By Western blotting employing these two affinity-purified antibodies, a singular band with an overall molecular mass of about 60 kD could be detected in human duodenal lysates (Fig. 1). Immunostaining of this polypeptide was completely abolished in the presence of 1$\mu$M immunogenic peptide. This band was not detectable when employing preimmune serum or the breakthrough of serum after affinity purification (Fig. 1). Additionally, the specificity of the two antibodies was proved by blotting against recombinant DMT1-protein (kindly provided by F. Kishi, Yamaguci University, Ube, Japan), where a pattern was observed similar to that previously described (not shown) (Tabuchi, 2000).

Distribution of DMT1 in human tissue. Because of the proposed role of DMT1 for duodenal iron absorption, expression of DMT1 in the intestine was investigated. The highest amounts of DMT1 were detected in duodenal epithelium, whereas in the epithelium of the esophagus, stomach, ileum and colon DMT1 was hardly detectable (Fig. 2). In the duodenum the highest expression of DMT1 was found in the villi (Fig. 4A). The staining was restricted to enterocytes, whereas goblet cells displayed no immunoreactivity. On the subcellular level DMT1 could be found mainly at the luminal surface of the epithelium, corresponding to the brushborder.

Furthermore, expression of DMT1 in gastrointestinal tissues with a known secretory function were investigated. In salivary glands such as the sublingual gland high amounts of DMT1 protein could be detected by Western blotting (Fig. 3). DMT1 was mainly found within the serous part of salivary glands and the luminal surface of intralobular and excretory ducts, while hardly any staining could be detected within mucous cells (Fig. 4B).

In the biliary system DMT1 was expressed by epithelial cells of the bile duct. Again, major amounts of DMT1 expression were detected on the luminal side of

cholangiocytes, which are responsible for secretion and absorption of divalent cations into and from the bile (Alpini et al., 2002). In accordance with this, high amounts of DMT1 were found at the luminal site of the gallbladder epithelium (Fig. 4E). Additionally, an increased expression of DMT1 was detected in human pancreas, where it was mainly localized in islet cells (Fig. 4C) and epithelial cells of pancreatic ducts (Fig. 4D).

To investigate whether DMT1 may be also highly expressed in other tissues with a putative transport function, human kidneys were studied. High amounts of DMT1 were found at the luminal site of the tubule system and over the entire length of the collecting ducts,

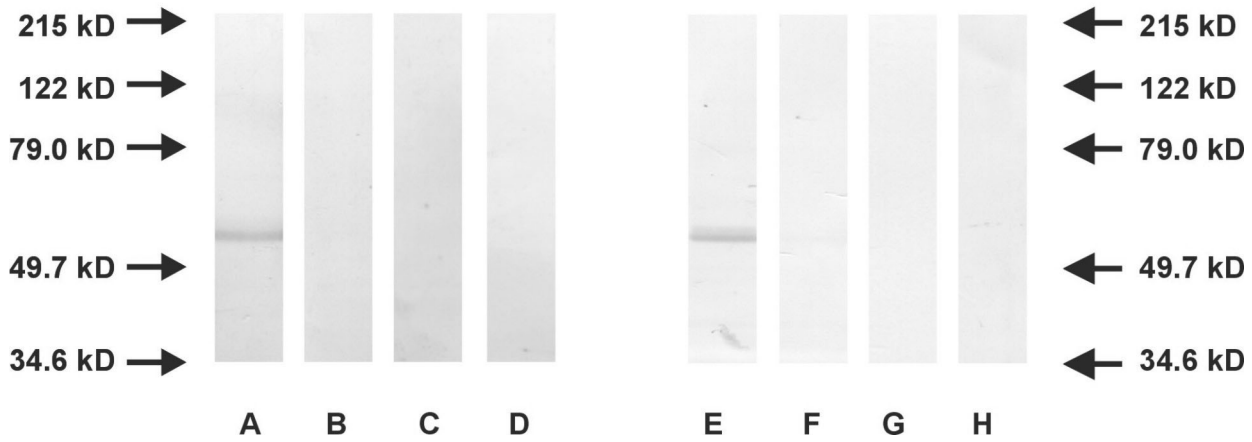


**Fig. 1.** Specificity of the anti-DMT1($_{174\text{-}187}$) and anti-DMT1($_{260\text{-}27}$5) antibodies: 15$\mu$g of duodenal tissue are separated on a 12% SDS-polyacrylamide gel and subsequently immunoblotted. In Lane A duodenal protein is stained with affinity-purified anti-DMT1($_{174\text{-}187}$) and in lane B with preimmune serum. In the presence of 1$\mu$M of the corresponding antigenic peptide staining of the DMT1 band is blocked (Lane C). Lane D: Incubation of Western blots with the breakthrough obtained from affinity purification of anti-DMT1($_{174\text{-}187}$) as described in Materials and Methods. In Lane E duodenal protein is stained with affinity-purified anti-DMT1($_{260\text{-}27}$5), in lane F with preimmune serum, in lane G in the presence of 1$\mu$M of the corresponding antigenic peptide and in lane H displays blotting with the breakthrough obtained from affinity purification of anti-DMT1($_{260\text{-}275}$).

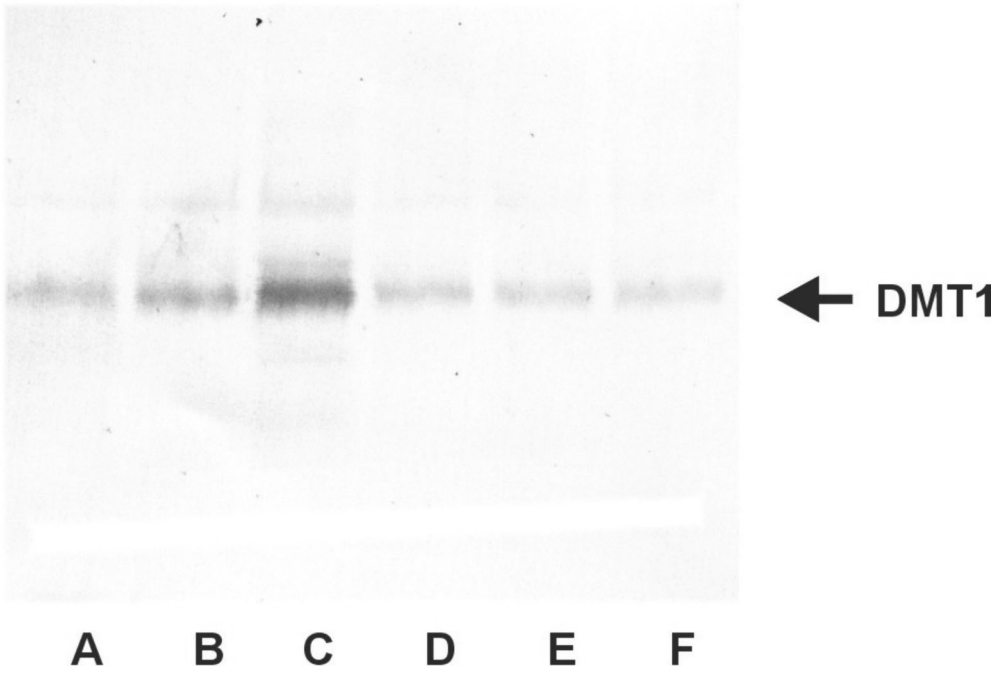


**Fig. 2.** Western blot analysis displays the quantitative distribution of DMT1 in human intestine: 20$\mu$g of protein obtained from esophagus (lane A), antrum (lane B), duodenum (lane C), terminal ileum (lane D), colon ascendens (lane E) and colon transversum (lane F) are stained employing anti-DMT1($_{174\text{-}187}$).

at a lower altitude and within the transitional epithelium of the renal pelvis, urether and bladder (Fig. 4F), while no DMT1 was found in renal glomeruli (Fig. 4G).

Within the respiratory system DMT1 expression was detected in epithelial cells throughout the trachea and the bronchial tree but it was most pronounced within the villous part. In a comparable fashion to our observations in the salivary glands positive staining for DMT1 was detected within the serous parts of the paratracheal glands (Fig. 4H).

## Discussion

In the study presented here, the pattern of expression and distribution of DMT1 in human tissue was analyzed. In the intestinal tract the expression of DMT1 was most prominent within the duodenal epithelium, which is in accordance with recently published data studying rat tissue (Trinder, 2000). While the crypt epithelium showed hardly any staining, high amounts of DMT1 were found on the luminal membrane of duodenal epithelial cells predominantly in villous enterocytes, whereas in the basolateral membrane of these cells no DMT1 was found. The localization of DMT1 in the brushborder of the duodenal enterocytes is in agreement with observations made in man and mice (Canonne-Hergaux, 999; Frazer, 2002; Zoller, 2001). The presence of DMT1 on the brushborder strongly supports the notion that DMT1 is important for uptake of ferrous iron from the lumen into enterocytes, but plays no role in basolateral export. Ferrous iron uptake by DMT1 requires previous reduction of ferric iron which is most likely exerted by a recently cloned duodenal membrane-associated ferric reductase named Dcytb (McKie, 2001). In contrast, basloateral iron export is exerted by a coordinated action of the recently identified iron transporter named ferroportin1 and the membrane-bound multicopper oxidase hephaestin (Donovan, 2000; McKie, 2000; Vulpe, 1999).

Although DMT1 initially appeared to be involved in the regulation of metal ion homeostasis including Fe2+, $Zn^{2+}$, $Cu^{2+}$, $Mn^{2+}$ or $Pb^{2+}$ throughout the body, the recent cloning and characterization of specific transport proteins for copper (HCTR1) and zinc (ZIP & ZNT1-7) has challenged this hypothesis (Harris, 2002; Sharp, 2003). Accordingly, like zinc transporters, which are known to be expressed in excretory glands, we also found a pronounced DMT1 expression in glandulae and excretory ducts of various organs. Its localization at the luminal site of the gallbladder and the biliary duct suggests that DMT1 is important for excretion or re-absorption of divalent cations from the bile. On the basolateral site no staining with anti-DMT1 antibodies could be found and it will be interesting to see whether or not ferroportin may be found in these tissues as well, which would then rather suggest that DMT1 may be responsible for re-absorption of divalent cations from the bile rather than for active excretion. This could be an important physiological mechanism for preventing loss of metal ions via biliary excretion.

As primary saliva, pancreatic juice or urine pass their respective duct system their ionic compositions are modified (Nauntofte, 1999). Thus, it is suggestive that the high expression of DMT1 in the biliary tract, the glandular system and the renal tubular system may participate in the regulation of the homeostasis of iron, zinc, copper and other divalent metals by modulating their excretion via urine, saliva and bile.

Interestingly, high amounts of DMT1 were found in islet cells of the pancreas. This may be of interest in the setting of hereditary hemochromatosis because DMT1 expression in the duodenum is highly upregulated

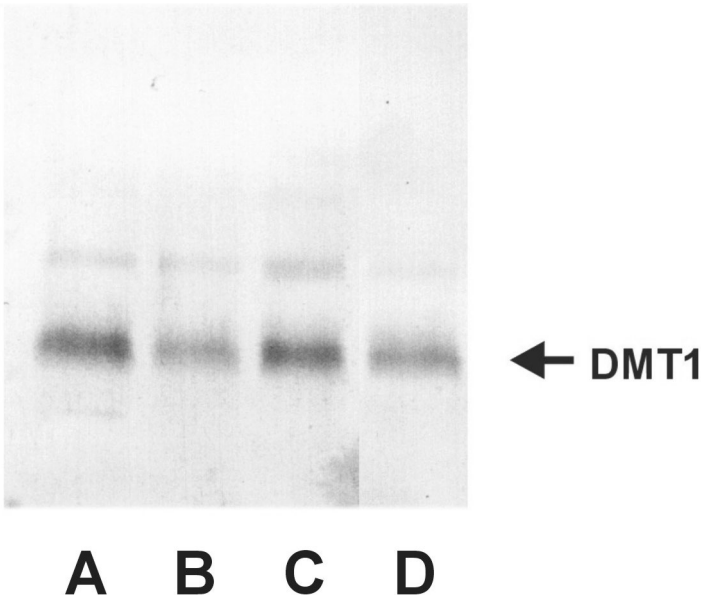


**Fig. 3.** Distribution of DMT1 in human tissues: Similar amounts of human tissue homogenates (20$\mu$g) are separated employing SDS-gel electrophoresis. DMT1 protein expression in the sublingual gland (A), kidney (B), gallbladder (C), and pancreatic tissue (D) is shown employing anti-DMT1$_{(174\text{-}187)}$.

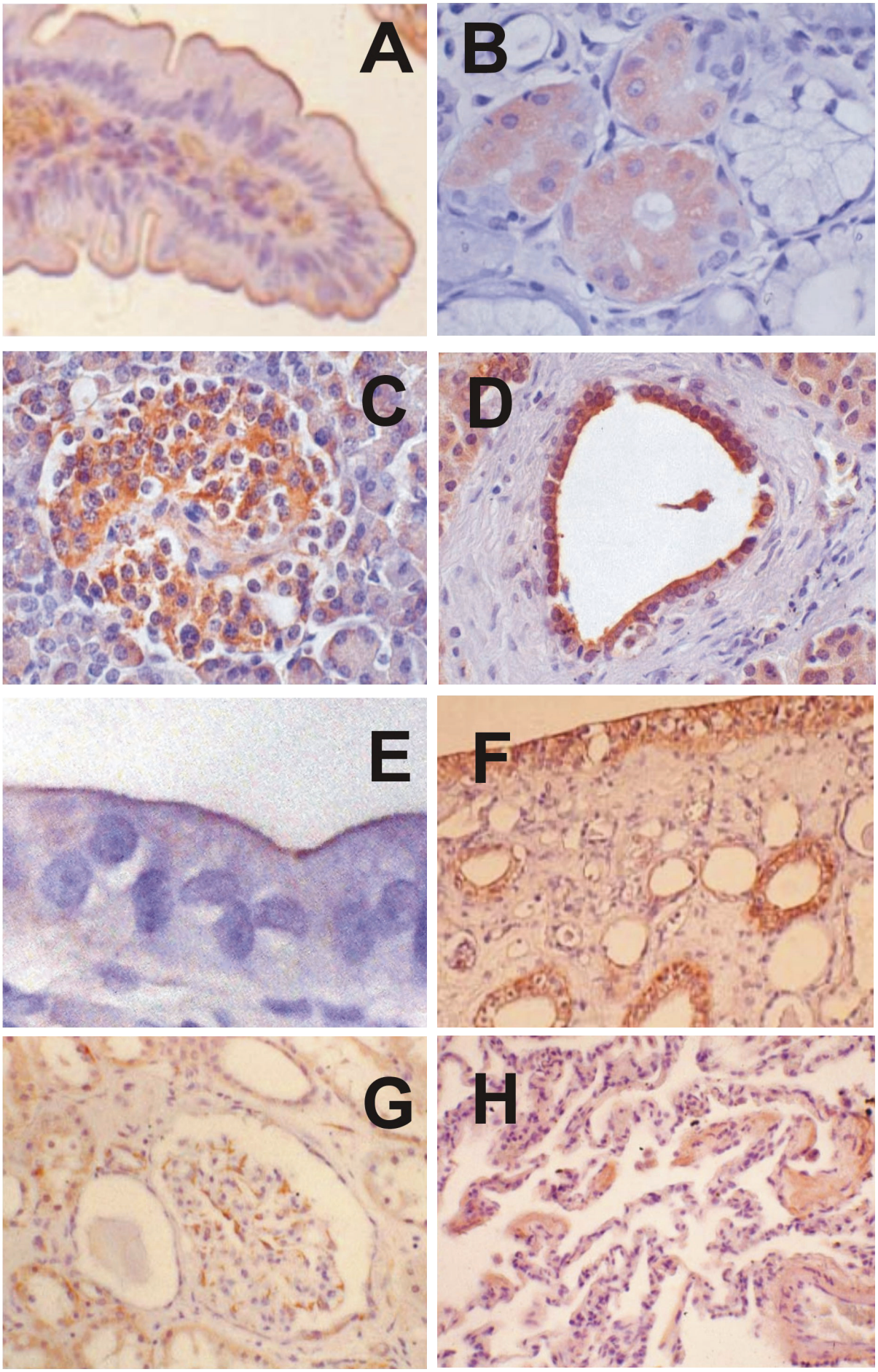


**Fig. 4.** DMT1 immuno-reactivity in different human tissues: In duodenum **(A)** the highest staining of DMT1 is detected in enterocytes, especially in the most luminal part. In the sublingual gland, staining is most pronounced within the serous part of salivary glands and the luminal surface of intercalated ducts **(B)**. **(C and D)** Pancreas. Immun-histochemical staining of DMT1 can be localized to islet cells **(C)** and to epithelial cells of pancreatic ducts **(D)**. In the gallbladder, labeling is most pronounced at the luminal site **(E)**. **(F and G)** Kidney. DMT1 can be detected in the tubule system and over the entire length of the collecting ducts. Urethral epithelium of renal pelvis also found on this section displays a diffuse staining pattern **(F)**. No staining was found in renal glomeruli **(G)**. **(H)** displays lung tissue with paratracheal glands. A, B, E-H, x 400. C, D, x 600

(Zoller, 2001) and because of the fact that a considerable amount of patients with hereditary hemochromatosis develop diabetes mellitus (Sheth and Brittenham, 2000). Although data on DMT1 expression in other tissues of patients with hereditary hemochromatosis have not been presented so far it is tempting to speculate that increased expression of DMT1 in islet cells may favor iron uptake into these cells and thus subsequent damage to islet cells via iron-mediated formation of toxic radicals (Ramos, 1992). This would fit to the observation that iron accumulation in pancreatic islet cells deteriorates pancreatic insulin secretion and leads to insulin-dependent diabetes mellitus which cannot be reversed by removal of iron (Niederau, 1999). The appearance of DMT1 in pancreatic islet cells seems to be a functional link in displaying a correlation between diabetes and hemochromatosis. Nevertheless, it will be interesting to study if DMT1 has a physiological function in islet cells especially towards insulin secretion.

In summary, we could demonstrate that DMT1 is most prominently expressed in the duodenum, which links DMT1 expression to metal ion absorption. Moreover, high amounts of DMT1 were found in various glandular tissues, the bile tract and the pancreas, thus providing evidence for a role of DMT1 in controlling the absorption / excretion of divalent metals within the body.

*Acknowledgements.* Dr. F. Kishi is gratefully acknowledged for providing recombinant DMT1-protein This work was supported by Grants from the Austrian Research Funds "FWF" P-15943 and from the Austrian National Bank P-8532 and P-8647.

## References

Alpini G., Baiocchi L., Glaser S., Ueno Y., Marzioni M., Francis H., Phinizy J.L., Angelico M. and Lesage G. (2002). Ursodeoxycholate and tauroursodeoxycholate inhibit cholangiocyte growth and secretion of BDL rats through activation of PKC alpha. Hepatology 35, 1041-1052.

Canonne-Hergaux F., Gruenheid S., Ponka P. and Gros P. (1999). Cellular and subcellular localization of the Nramp2 iron transporter in the intestinal brush border and regulation by dietary iron. Blood 93, 4406-4417.

Donovan A., Brownlie A., Zhou Y., Shepard J., Pratt S.J., Moynihan J., Paw B.H., Drejer A., Barut B., Zapata A., Law T.C., Brugnara C., Lux S.E., Pinkus G.S., Pinkus J. L., Kingsley P.D., Palis J., Fleming M.D., Andrews N.C. and Zon L.I. (2000). Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. Nature 403, 776-781.

Fleming M. D., Trenor C.C., III, Su M. A., Foernzler D., Beier D.R., Dietrich W. F. and Andrews N.C. (1997). Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. Nat.Genet. 16, 383-386.

Frazer D.M., Wilkins S.J., Becker E.M., Vulpe C.D., McKie A.T., Trinder D. and Anderson G.J. (2002). Hepcidin expression inversely correlates with the expression of duodenal iron transporters and iron absorption in rats. Gastroenterology 123, 835-844.

Gunshin H., Mackenzie B., Berger U.V., Gunshin Y., Romero M.F., Boron W.F., Nussberger S., Gollan J.L. and Hediger M.A. (1997). Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature 388, 482-488.

Gunshin H., Allerson C. R., Polycarpou-Schwarz M., Rofts A., Rogers J. T., Kishi F., Hentze M.W., Rouault T.A., Andrews N.C. and Hediger M.A. (2001). Iron-dependent regulation of the divalent metal ion transporter. FEBS Lett. 509, 309-316.

Harris E.D. (2002). Cellular transporters for zinc. Nutr.Rev. 60, 121-124.

Hentze M.W. and Kuhn L.C. (1996). Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. Proc. Natl. Acad. Sci. USA 93, 8175-8182.

Kishi F. and Tabuchi M. (1997). Complete nucleotide sequence of human NRAMP2 cDNA. Mol. Immunol. 34, 839-842.

Koch R., Trieb M., Koschak A., Wanner S., Gauthier K., Rusch N., and Knaus H. (2001). Design and use of antibodies for mapping K+ channel expression in cardiovascular system. In: Potassium channels in cardiovascular biology. 1rd ed. Archer S.L. and Rusch N. (eds). Kluwer Academic/Plemum Publisher. New York, Boston, Dordrecht, London, Moscow. pp 91-102.

Lee P.L., Gelbart T., West C., Halloran C. and Beutler E. (1998). The human Nramp2 gene: characterization of the gene structure, alternative splicing, promoter region and polymorphisms. Blood Cells Mol.Dis. 24, 199-215.

McKie A.T., Marciani P., Rolfs A., Brennan K., Wehr K., Barrow D., Miret S., Bomford A., Peters T.J., Farzaneh F., Hediger M.A., Hentze M.W. and Simpson R.J. (2000). A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. Mol. Cell 5, 299-309.

McKie A.T., Barrow D., Latunde-Dada G.O., Rolfs A., Sager G., Mudaly E., Mudaly M., Richardson C., Barlow D., Bomford A., Peters T.J., Raja K.B., Shirali S., Hediger M.A., Farzaneh F. and Simpson R.J. (2001). An iron-regulated ferric reductase associated with the absorption of dietary iron. Science 291, 1755-1759.

Nauntofte B. (1999b). Salivary secretion. In: Textbook of gastroenterology. 3 rd ed. Yamada T., Alpers D., Laine L., Owyamg C., and Powell D .(eds). Lippincott Williams & Wilkins. Phildaelphia, New York, Baltimore. pp 263-277.

Niederau C. (1999). Diabetes mellitus in hemochromatosis. Z.Gastroenterol. Suppl 1, 22-32.

Ramos C.L., Pou S., Britigan B.E., Cohen M.S. and Rosen G.M. (1992). Spin trapping evidence for myeloperoxidase-dependent hydroxyl radical formation by human neutrophils and monocytes. J. Biol. Chem. 267, 8307-8312.

Rouault T. and Klausner R. (1997). Regulation of iron metabolism in eukaryotes. Curr. Top. Cell Regul. 35, 1-19.

Sharp P.A. (2003). Ctr1 and its role in body copper homeostasis. Int. J.. Biochem. Cell Biol. 35, 288-291.

Sheth S. and Brittenham G. M. (2000). Genetic disorders affecting proteins of iron metabolism: clinical implications. Annu. Rev. .Med. 51, 443-464.

Su M.A., Trenor C.C., Fleming J.C., Fleming M.D. and Andrews N.C. (1998). The G185R mutation disrupts function of the iron transporter Nramp2. Blood 92, 2157-2163.

Tabuchi M., Yoshimori T., Yamaguchi K., Yoshida T. and Kishi F. (2000). Human NRAMP2/DMT1, which mediates iron transport across endosomal membranes, is localized to late endosomes and lysosomes in HEp-2 cells. J.Biol.Chem. 275, 22220-22228.

Tchernitchko D., Bourgeois M., Martin M. E., and Beaumont C. (2002).

Expression of the two mRNA isoforms of the iron transporter Nrmap2/DMTI in mice and function of the iron responsive element. Biochem.J. 363, 449-455.

Trinder D., Oates P. S., Thomas C., Sadleir J. and Morgan E. H. (2000). Localisation of divalent metal transporter 1 (DMT1) to the microvillus membrane of rat duodenal enterocytes in iron deficiency, but to hepatocytes in iron overload. Gut 46, 270-276.

Vulpe C. D., Kuo Y. M., Murphy T. L., Cowley L., Askwith C., Libina N., Gitschier J. and Anderson G. J. (1999). Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. Nat. Genet. 21, 195-199.

Zoller H., Koch R.O., Theurl I., Obrist P., Pietrangelo A., Montosi G., Haile D. J., Vogel W. and Weiss G. (2001). Expression of the duodenal iron transporters divalent-metal transporter 1 and ferroportin 1 in iron deficiency and iron overload. Gastroenterology 120, 1412-1419.

Zoller H., Theurl I., Koch R., Kaser A. and Weiss G. (2002). Mechanisms of iron mediated regulation of the duodenal iron transporters divalent metal transporter 1 and ferroportin 1. Blood Cells Mol. .Dis. 29, 488-497.

Accepted May 23, 2003