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HALOTHANE METABOLISM: KUPFFER CELLS CARRY AND PARTIALLY PROCESS TRIFLUOROACETYLATED PROTEIN ADDUCTS

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SUMMARY: Kupffer cells, prepared 18 h after pretreatment of rats with a single dose of halothane, did carry TFA-adducts which were recognized on Western blots by a anti-TFA-antibody. Based on apparent molecular weight, the pattern of the major TFA-adducts within Kupffer cells was similar to that observed in hepatocytes. When kept in primary culture, Kupffer cells processed TFA-adducts of apparent molecular weight of 220 kD, 110 kD and 74 kD within 24 or 48 h; in contrast, other TFA-adducts were persistent for at least 48 h in Kupffer cells. The data suggest a role for Kupffer cells in processing of chemically altered proteins in the liver.

Fulminant massive liver necrosis ("halothane hepatitis") is the more severe form of hepatotoxicity associated with metabolism of the anesthetic agent halothane. Upon single or repeated exposure to the anesthetic, ~ 1 in 30000 or 1 in 3000 patients are afflicted, respectively (1). There is ample evidence suggesting that halothane hepatitis has an immunological basis (2). Most convincingly, sera of halothane hepatitis patients, but not of according control individuals, contain antibodies against prominent rat liver microsomal heterologous polypeptides of ~ 100 kD, 76 kD, 59 kD, 57 kD and 54 kD. These polypeptides are trifluoroacetylated at primary amine groups by the reactive halide CF_3COCl which arises as a major metabolite upon oxidative, cytochrome P450-dependent metabolism of halothane (i.e., so-called TFA-adducts[1] (3,4)). Such TFA-adducts have previously been detected on the surface of isolated rat hepatocytes (5), in rat and rabbit liver microsomal fractions (6,7), and in guinea pig centrilobular liver sections (8). Moreover, limited studies seemed to indicate that liver biopsies obtained from halothane hepatitis patients also contained such TFA-adducts (9).

To date, there is nothing known about mechanisms that lead to the recognition of endogenously generated TFA-adducts by competent cells of the immune system; at one stage, such TFA-adducts or processed fragments thereof would have to be presented in a MHC class I or class II restricted manner by antigen presenting cells (10, 11). However, no "professional" antigen presenting cell (APC) has been identified so far that carries TFA-adducts. We have speculated that in the liver, resident macrophages (i.e. Kupffer cells), naturally competent for presenting circulating antigens (12), might be involved in processing and/or presenting of TFA-adducts or fragments thereof. Such a scenario would minimally require that Kupffer cells acquire endogenously generated

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[1] The term "TFA-adduct" denotes polypeptides carrying trifluoroacetylated amino acid residues.

Abbreviations: TBS, Tris-buffered saline; PBS, Phosphate-buffered saline; HRP, horseradish peroxidase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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TFA-adducts from neighboring hepatocytes or, alternatively, are being capable of TFA-adduct formation themselves. Here, using Western blotting techniques, we present evidence that Kupffer cells obtained from halothane-treated rats do carry distinct TFA-adducts as recognized by a monoform anti-TFA-antibody. Based on apparent molecular weight, the pattern of the major TFA-adducts recognized in Kupffer cells was similar to that observed in hepatocytes. When kept in primary culture, Kupffer cells did process[2] some of these TFA-adducts within 24 and 48 h whereas others were persistent for at least 48 h. These data suggest a role for Kupffer cells in processing of chemically altered polypetides in the liver.

METHODS

Treatment of Animals

Male Sprague-Dawley rats (250 -300g) were pretreated with halothane (10 mmol/kg body weight, as a 50% (v/v) solution in sesam oil) at the specified period of time before cells were prepared. Control animals did not receive halothane. When hepatocytes only were obtained, rats were pretreated first with three daily injections (i.p.) of 80 mg/kg body weight of sodium phenobarbital in PBS prior to the halothane treatment.

Preparation of hepatocytes

Animals were fasted for 12 h prior to the experiment. After animals were anesthetized (50 mg/kg body weight pentobarbital (Nembutal), i.p.), single cell suspensions of hepatocytes were prepared by in situ perfusion with collagenase/dispase as described (13, 14). Separation of hepatocytes from non-parenchymal liver cells was performed according to Smedsrød and Pertoft (13) with slight modifications. Briefly, cell suspensions were filtered through nylon mesh and then centrifuged for 4 min at 30 x g, yielding a pellet enriched in hepatocytes and a supernatant fraction enriched in non-parenchymal cells. The pellet was gently resuspended in PBS, pH 7.4, and aliquots of 20 ml were immediately layered on top of a Percoll cushion (15 ml) with a density of 1.070 g/ml and centrifuged at 130 x g for 10 min. Viable hepatocytes (by Trypan blue exclusion), which only penetrated the Percoll layer, were collected and washed once more with PBS. Hepatocytes prepared by passage through a Percoll layer of a density of 1.070 g/ml typically contained less than 1% Kupffer cells (as tested by peroxidase staining). Finally, the obtained pellet of hepatocytes was resuspended in TBS, containing 0.5 mM PMSF and 60 µg/ml soybean trypsin/chymotrypsin inhibitor, and a homogenate was prepared by disruption of hepatocytes in TBS by four strokes in a Potter Elvehjem homogenizer at 4°C followed by sonication for 10 min in a bath type sonifier (Branson) in an ice-water bath. The homogenates were stored in aliquots at - 80 °C and typically thawed only once just prior to the experiment. Protein concentrations in all experiments were estimated using the Bio-Rad procedure with BSA as a standard.

Preparation of Kupffer cells

Kupffer cells were obtained by means of Percoll-gradient enrichment and selective adherence to plastic (13). Briefly, the fraction of non-parenchymal cells obtained after the low speed sedimentation of heptocytes (see above) was applied in aliquots (10 ml) onto a pre-formed step gradient of Percoll comprising a lower and upper cushion (10ml each) with a density of 1.066 g/ml and 1.037 g/ml, respectively. Gradients were centrifuged at 800 x g for 20 min. Kupffer cells, which were spread throughout the 1.066 g/ml cushion and quantitatively separated from erythrocytes, liver endothelial cells and non-viable cell debris, were freed of Percoll and transferred into RPMI-1640 culture medium (GIBCO). These cells were then plated on 10 x 35 mm plastic culture dishes (Costar) at a density of 5 x10^6 cells/plate. Cells were allowed to adhere for 20 min at 37 °C in a humidified atmosphere of air/CO_2 (95%/5%, v/v); after 20 min incubation, cells that did not adhere were removed through vigorous rinsing (2 x) of the culture dishes with medium. Viability of adherent cells (by Trypan blue exclusion) was > 95 %; cells were identified as Kupffer cells by peroxidase staining and ingestion of latex particles (0.8 µm). At that point, > 99 % of cells were Kupffer cells; they were harvested by scrapping off the plate with a rubber policeman and homogenized as described for hepatocytes. In some experiments, Kupffer cells were cultured in RPMI-1640 culture medium for 24 or 48 h at 37 °C in a humidified atmosphere of air/CO_2 (95%/5%, v/v); cells were harvested followed by preparation of cell homogenates and analysis on Western blots as described.

Preparation of affinity-purified anti-TFA-antibody

Polyclonal anti-TFA-RSA sera were obtained adhering to published procedures (5) from female New Zealand white rabbits after initial immunization with trifluoroacetylated rabbit serum albumin (TFA-RSA, 2 mg emulsified in complete Freund's adjuvant) followed by three weekly booster injections (2 mg TFA-RSA emulsified in incomplete Freund's adjuvant). A total IgG-fraction was obtained through precipitation (2x) of sera (10 ml) with 40 % (w/v) ammonium sulfate. Total IgG was resuspended in PBS, containing 0.75 M NaCl and 1% BSA (w/v), and preadsorbed on an Affi-Gel 102 amino terminal agarose gel matrix (Bio-Rad) in order to remove IgG nonspecifically binding to the gel matrix. The remainder was applied to an Affi-Gel 102 amino

[2] The term "processing" is used here to indicate any mechanism leading to a decrease in recognition by anti-TFA-antibodies of TFA-adducts.

terminal agarose column (1.0 x 10 cm), to which N-ε-TFA-L-lysine had been coupled by carbodiimide-facilitated chemistry according to the manufacturers guidelines (Bio-Rad) and which had been equilibrated with PBS, containing 0.75 M NaCl and 1% BSA (w/v). The IgG-containing solution (10 ml) was recirculated over the column at 10 ml/h for 16 h followed by washing the column with PBS, containing 0.75 M NaCl, until $\Delta OD_{280\ nm}$ returned to basis level; anti-TFA-antibody was then eluted with 100 mM N-ε-TFA-L-lysine in PBS, containing 0.75 M NaCl. Fractions containing material absorbing at 280 nm were collected and tested for reactivity towards TFA-RSA on Western blots. Reactive fractions were pooled, supplemented with 1% BSA (w/v) and 0.05% sodium azide and dialyzed at 4°C against a 10^{12} -fold volume of PBS containing 0.05% sodium azide. Aliquots were stored at -80°C and typically thawed only once.

Gel electrophoresis and Western blotting

Hepatocyte or Kupffer cell homogenates (5-10 mg protein/ml) were, after sonication, mixed 1:1 (v/v) with dissociation buffer (12 mM Tris-HCl, pH 6.8, 8% SDS (w/v), 10% glycerol, 40 mM DTT) and heated to 95 °C for 10 min. SDS-PAGE was performed using a 4.5 % stacking and a 10% separating gel. Except where noted, protein loading was 250 µg/cm slot width. Electrophoresis was for 4 h at 30 mA/gel. Resolved polypeptides were transferred electrophoretically to nitrocellulose at 360 V x h using a transfer buffer comprising 15.6 mM Tris, 120 mM glycine and 20% methanol (v/v) at pH 8.3. The nitrocellulose was stained with amido black for visualizing proteins, destained, and blocked for 2 h at room temperature with PBS containing 2% (w/v) dry milk powder and 0.02% (w/v) Thimerosal. Strips (3 mm width) were then used for antibody overlay. Incubation with the first antibody (appropriately diluted into blocking solution) was for 18 h at room temperature under constant shaking. After 5 washes of 5 min each with blocking solution, incubation with HRP-conjugated second antibody (diluted into blocking solution) was for 2 h at room temperature. After one wash with blocking solution and four washes with PBS for 5 min each, peroxidase activity was developed with 4-chloro-1-naphtol as a substrate. When Western blots derived from Kupffer cell homogenates were developed, the enhanced chemiluminescence detection system (ECL, Amersham) was used according to the manufacturers guidelines. In all experiments, apparent molecular weights of antigenic polypeptides were estimated by comparing their relative mobilities to that of marker proteins of known molecular weight.

RESULTS AND DISCUSSION

Ample evidence suggests that halothane-induced hepatitis, a rare but severe form of an idiosyncratic drug reaction associated with the anesthetic, has an immunological basis (2). Oxidative metabolism of halothane ($CF_3CHClBr$) by liver microsomal cytochrome P450 yields, among other products, the reactive halide intermediate CF_3COCl (15), which readily reacts with cellular macromolecules (i.e., proteins, phospholipids and RNA and/or DNA as presumable targets) giving rise to so-called TFA-adducts (3). Sera of patients afflicted with halothane-induced hepatitis, but not those of according control individuals, exhibit, when tested on Western blots against heterologous rat liver microsomal triflouroacetylated polypeptides, antibodies directed against TFA-adducts, preferentially with apparent molecular weight of ~ 100 kD, 76 kD, 59 kD 57 kD and 54 kD (3). Some of these polypeptides have been purified and cloned (16, 17); however, so far, nothing is known about the true nature of (a) presumable neo-immunogen(s) arising in susceptible individuals upon exposure to halothane. Moreover, the initial steps (i.e. processing, presentation) by which endogenously generated TFA-adducts or fragments thereof could become exposed to competent cells of the immune system have not been elucidated.

In the liver, resident macrophages (i.e., Kupffer cells) might be a candidate cell type to be involved in this process. In this report, we provide evidence that Kupffer cells, obtained from livers of rats pretreated with halothane, in fact do carry TFA-adducts. When probed for on Western blots with a monoform anti-TFA-antibody followed by detection with enhanced chemiluminescence, homogenates of Kupffer cells, obtained from rats 18 h after exposure to a single dose of halothane (10 mmol/kg body weight), exhibited trifluoro-acetylated protein adducts of apparent molecular weight of about 60 kD, 62 kD, 74 kD, 78 kD, 98 kD, 105 kD, 110 kD, 140 kD, and 220 kD (Fig. 1, lane 4). The pattern of the major TFA-adducts detected in Kupffer cell homogenates (based on apparent molecular weight) was similar to that observed in homogenates of hepatocytes (Fig. 1, lane 2) obtained from the same animal at the same time point (i.e., 18 h after exposure to halothane); hepatocytes did exhibit, however, a number of additional TFA-adducts. In control experiments both Kupffer

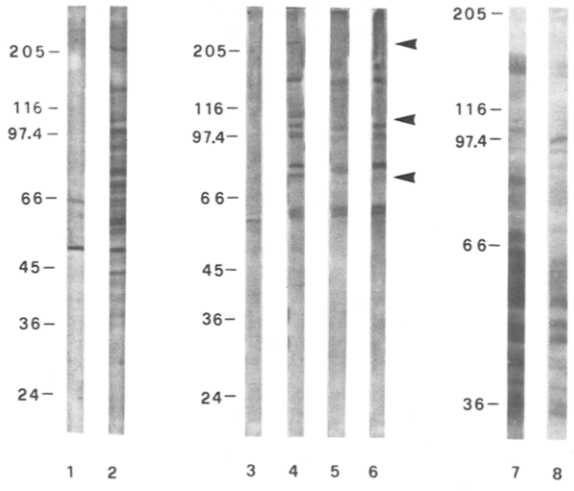

Figure 1. Detection of TFA-adducts on Western blots of Kupffer cells - Rats were treated with a single dose of halothane (10 mmol/kg body weight). Kupffer cells and hepatocytes were obtained through in situ collagenase/dispase perfusion of the liver. SDS-PAGE of Kupffer cell (250 µg/cm slot width) and hepatocyte (250 µg/cm slot width) homogenates was followed by visualizing of TFA-adducts on Western blots using, sequentially, a monoform anti-TFA-antibody, a HRP-conjugated second antibody, and enhanced chemiluminescence detection (ECL). Lane 1: Hepatocytes of a control rat (not exposed to halothane); Lane 2: Hepatocytes prepared 18 h after exposure to halothane; Lane 3: Kupffer cells of a control rat; Lane 4: Kupffer cells prepared 18 h after exposure to halothane; Lanes 5 and 6: Kupffer cells (prepared 18 h after exposure to halothane), cultured for 24 h and 48 h, respectively; Lanes 7 and 8: Amido-black staining on nitrocellulose of total proteins of hepatocytes and Kupffer cells (each prepared 18 h after exposure to halothane), respectively, prior to probing with anti-TFA-antibody. Hepatocytes (lane 1) and Kupffer cells (lane 3) were from a single control rat; hepatocytes (lane 2) and Kupffer cells (lanes 4 and 5) were also prepared from a single rat 18 h after exposure to halothane. The relative mobilities on SDS-PAGE of proteins of known molecular weight are indicated (myosin, rabbit muscle subunit (205 kD); β-galactosidase, E. coli (116 kD); phosphorylase b, rabbit muscle subunit (97.4 kD); albumin, bovine plasma (66 kD); albumin, egg ovalbumin (45 kD); pepsin, porcine stomach mucosa (36 kD); trypsinogen, bovine pancreas (24 kD)). Arrow heads indicate location of processed TFA-adducts.

cell and hepatocyte homogenates, obtained from rats 18 h after exposure to a single dose of halothane, were treated with piperidine (1 mM) in order to cleave trifluoroacetyl groups (16). No TFA-adducts were recognized by the anti-TFA-antibody on Western blots of those cells (data not shown). These data confirmed that the adducts recognized on Western blots of both Kupffer cells and hepatocytes in fact were polypeptides modified through trifluoroacetylation. Surprisingly, on Western blots of hepatocyte homogenates, obtained from control animals that had not received halothane, monoform anti-TFA-antibodies did recognize a 52 kD, and, to a much lesser degree, a 64 kD crossreactive polypeptide (Fig. 1, lane 1). The recognition of these polypetides was not sensitive to piperidine treatment (data not shown); to date, nothing is known about the basis of the crossreactivity of these polypeptides with the anti-TFA-antibody.

Kupffer cells did not only carry but were also capable of processing TFA-adducts. In according experiments, Kupffer cells were cut from a possible supply of TFA-adducts, arising in vivo from intra- or inter-cellular stores, by taking cells, obtained 18 h after exposure of rats to a single dose of halothane, into primary culture for 24 or 48 h. As illustrated in Fig. 1, lanes 5 and 6, recognition by anti-TFA-antibody of TFA-adducts with apparent molecular weight of about 220 kD, 110 kD, and 74 kD (arrow heads) decreased or was no longer evident on Western blots of Kupffer cells after 24 or 48 h of primary culture, respectively. Contrastingly, TFA-adducts with apparent molecular weight of about 140 kD, 105 kD, 98kD, 78 kD, 62 kD, and 60 kD were persistent within a time period of 48 h with only marginal decrease in recognition by anti-TFA-antibody. These findings suggest that Kupffer cells do process TFA-adducts; the processing seems to be differential on the

temporal axis. The extent of processing may possibly depend on **i)** the nature of the respective TFA-adducts, **ii)** the protease activities involved which may express distinct affinities for individual TFA-adducts and, **iii)** the subcellular localization of TFA-adducts which might limit accessibility of TFA-adducts to according proteases. In these experiments, we have not yet been able to identify by Western blotting low molecular weight fragments (peptides) of the processed TFA-adducts.

Processing of TFA-adducts is not restricted to Kupffer cells but also found in hepatocytes in vivo. Examination of Western blots of homogenates prepared from rat hepatocytes obtained 18 h, 42 h, 96 h or 10 days after exposure of rats to a single dose of halothane revealed a most intense staining of TFA-adducts after 18 h; the intensity of staining of recognized polypeptides was significantly decreased after 42 h and 96 h. Slight variations were observed in the time after which TFA-adducts were still recognized by the anti-TFA-antibody. Most prominently, the recognition of a polypeptide of apparent molecular weight of ~48 kD was already abolished after 42 h, whereas the other TFA-adduts were persistent for at least 96 h but not longer than 10 days. In fact, 10 days after exposure to halothane, no TFA-adducts were recognizable; however, a 52 kD crossreactive polypeptide (and, very faintly, a 64 kD crossreactive polypeptide) were still recognized (Fig. 2). This finding suggests that the crossreactive polypeptides seem not to be subjected to any of the mechanisms that lead in hepatocytes in vivo to the disappearance of TFA-adducts. It is interestingly to note that also in Kupffer cells, obtained 10 days after halothane exposure and analyzed on Western blots immediately (i.e., without prior culture), no TFA-adducts were detectable (data not shown).

The data presented here clearly indicate that in the liver, both Kupffer cells and hepatocytes are involved in processing of polypeptides endogenously modified through adduct formation. Nothing is known at present about the basis of the striking similarity in apparent molecular weight of some of the TFA-adducts found in

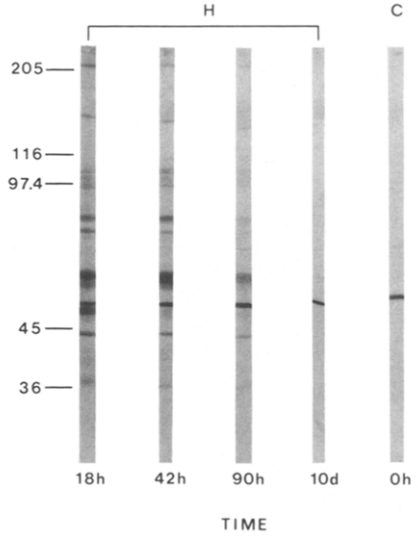

Figure 2. Persistence of TFA-adducts in rat hepatocyte homogenates - Prior to administration of a single dose of halothane (10 mmol/kg body weight), rats were pretreated by three daily injections (i.p.) of 80 mg/kg body weight of sodium phenobarbital in PBS. Hepatocytes were obtained through in situ collagenase/dispase perfusion of the liver. SDS-PAGE of hepatocyte homogenates (250 µg/cm slot width) was followed by visualizing of TFA-adducts on Western blots using, sequentially, a monoform anti-TFA-antibody, a HRP-conjugated second antibody and development of peroxidase activity with 4-chloro-1-naphtol. H: Hepatocytes prepared 18 h, 42 h, 90 h, and 10 days after exposure to halothane. C: Hepatocytes prepared from a control rat not exposed to halothane (0 h). The relative mobilities on SDS-PAGE of proteins of known molecular weight are indicated (myosin, rabbit muscle subunit (205 kD); β-galactosidase, E. coli (116 kD); phosphorylase b, rabbit muscle subunit (97.4 kD); albumin, egg ovalbumin (45 kD); pepsin, porcine stomach mucosa (36 kD)).

both Kupffer cells and hepatocytes. The polypeptide outfit of Kupffer cells and hepatocytes is as such that some polypeptides may be found in both cell types (19) whereas others may be cell specific. An amido-black staining of resolved proteins on nitrocellulose, just before probing Western blots with the anti-TFA-antibody, did reveal recognizable differences in the pattern obtained depending on whether Kupffer cells (Fig. 1, lane 8) or hepatocytes (Fig. 1, lane 7) were analyzed. The apparently identical molecular weight of some TFA-adducts found in both Kupffer cells and hepatocytes might point to the possibility that these TFA-adducts have the same cellular origin. Hepatocytes would be expected to be mostly responsible for drug metabolism, including that of halothane. Given the high reactivity of the halide intermediate CF_3COCl, one would expect trifluoroacetylation of polypeptides primarily located close to the site of generation of the reactive metabolite. The question arises then how Kupffer cells could acquire TFA-adducts. The present study does not allow to discriminate the following three possibilities. First: The halide intermediate CF_3COCl is sufficiently long lived to diffuse away from the site of generation and reacts with particularly susceptible polypeptides in Kupffer cells. Such polypeptides are either incidentally identical in electrophoretic mobility to polypeptides found in hepatocytes, or, alternatively, are in fact identical proteins occuring in both Kupffer cells and hepatocytes. Second: Trifluoro-acetylation tags hepatocytes for phagocytotic uptake by Kupffer cells. TFA-adducts found associated with Kupffer cell homogenates therefore are transferred from hepatocytes. Third: Kupffer cells have the capacity of metabolizing halothane and consequently, TFA-adducts are arising from within Kupffer cells. This last possibility is corroborated by studies of Steinberg et. al. which have revealed that xenobiotic metabolizing enzymes are not restricted to liver parenchymal cells (19). Kupffer cells were shown to express, as demonstrated both by functional tests and immunochemically, cytochrome $P450_{PB-4}$, $P450_{MC1a}$, $P450_{MC1b}$ and microsomal epoxide hydrolase. However, to our knowledge, no halothane metabolism by Kupffer cells has been demonstrated so far.

Clearly, further experimentation will be needed to clarify the mechanisms by which Kupffer cells acquire TFA-adducts as well as the ultimate fate of these adducts within Kupffer cells. If Kupffer cells are involved in the process of presenting fragments of TFA-adducts, one might expect to find naturally processed peptides of rather uniform size (20, 21, 22) carrying the trifluoroacetyl group associated with the polypeptides of the MHC. If, on the other hand, the role of Kupffer cells is limited to degradative removal of chemically modified proteins, characteristic amino acid derivatives may be products of this degradation. In fact, N^2-acetyl-N^6-DNP-lysine was identified as both a biliary and an urinary metabolite arising in rats upon in vivo degradation of i.v. administered rat and human serum albumin conjugated with the dinitrophenyl hapten (RSA-DNP and HSA-DNP, respectively (23)). In contrast to these experiments, using one particular exogenously prepared model drug-protein adduct, the study presented here identifies initial steps within the liver in the fate of the multitude of distinct endogenous TFA-adducts generated in vivo.

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