Loss of Cell Membrane Integrity in Puumala Hantavirus-Infected Patients Correlates with Levels of Epithelial Cell Apoptosis and Perforin

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Hantaviruses are the causative agents of hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). Capillary leakage is a hallmark of hantavirus infection. Pathogenic hantaviruses are not cytotoxic, but elevated levels of serum lactate dehydrogenase (LDH), indicative of cellular damage, are observed in patients. We report increased levels of serum perforin, granzyme B, and the epithelial cell apoptosis marker caspase-cleaved cytokeratin-18 during Puumala hantavirus infection. Significant correlation was observed between the levels of LDH and perforin and the levels of LDH and caspase-cleaved cytokeratin-18, suggesting that tissue damage is due to an immune reaction and that epithelial apoptosis contributed significantly to the damage.

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showing that the highest levels of perforin and granzyme B were detected early after the onset of fever (Fig. 1C).

The levels of perforin and granzyme B in the individual patients during the acute phase also correlated (Spearman R = 0.56; P = 0.015) (Fig. 1D).

Consistent with previous observations (3, 5, 37), all patients in this study showed increased levels of serum LDH during the acute phase of infection. The LDH levels did not, however, correlate significantly (Spearman R = −0.30; P = 0.24) with time after onset of fever (Fig. 2). The level of LDH correlated significantly with that of perforin (Spearman R = 0.50; P = 0.039) (Fig. 3) but not with the levels of granzyme B (Spearman R = 0.27; P = 0.30).

To quantify possible epithelial cell apoptosis in hantavirus-infected patients, we determined the serum levels of a caspase cleavage product of CK18 (1). CK18 is a type I intermediate filament protein that is exclusively expressed by simple epithelial cells, like those of the endothelia. CK18 is cleaved by caspases during apoptosis and is released into serum (1, 2). Increased serum levels of caspase-cleaved CK18 have previously been detected in patients during septic shock (28), in patients with hepatitis (16), and in patients with various carcinomas (19). Interestingly, significantly higher levels of caspase-cleaved CK18 were observed during the acute phase of infection than during the convalescent phase (P = 0.0028 by Wilcoxon signed-rank test) (Fig. 4A). Fourteen of the 18 patients showed higher levels of caspase-cleaved CK18 during the acute phase than during the convalescent phase. Similar to serum LDH, the level of epithelial cell apoptosis showed no

convalescent (9.8 ± 3.1 ng perforin/ml, ranging from 5.4 to 15.0 ng/ml) phases of HFRS are shown. All but two samples were negative for granzyme B during the convalescent phase; the two positive samples had 4.4 and 13.3 pg granzyme B/ml, respectively. Data represent means ± standard deviations (n = 18). (C) Levels of perforin and granzyme B in serum for the acute-phase samples plotted against days after initial fever for the individual patients. (D) Levels of perforin plotted against the level of granzyme B in acute-phase serum from the individual patients. The serum levels of perforin and granzyme B in healthy subjects has been reported to be 8.0 ± 2.79 ng perforin/ml (15) and 11.5 pg granzyme B/ml (32), respectively.
significant correlation (Spearman $R = -0.45; P = 0.059$) with time after onset of fever.

The level of caspase-cleaved CK18 correlated significantly with that of LDH (Spearman $R = 0.74; P = 0.00067$) (Fig. 4B). No significant correlation was observed between the levels of perforin (Spearman $R = 0.40; P = 0.10$) or granzyme B (Spearman $R = 0.29; P = 0.24$) with those of caspase-cleaved CK18.

The observed elevated levels of caspase-cleaved CK18 show that apoptosis is induced in cells of the epithelial cell lineage during the acute phase of hantavirus infection. Furthermore, the very strong correlation between the levels of epithelial apoptosis and LDH suggests that most of the cell damage observed during hantavirus infections is caused by apoptosis. To our knowledge, this is the first report showing apoptosis of epithelial cells during hantavirus infection in humans.

Significantly higher levels of perforin and granzyme B were observed during the acute phase than during the convalescent phase of infection. Furthermore, the levels of perforin and granzyme B correlated during the acute phase. It could be speculated that the levels of perforin and granzyme B in released cytotoxic granules are roughly proportional to each other during acute infection.

The levels of perforin and LDH correlated significantly, suggesting that hantavirus-specific CD8$^+$ T cells and/or NK cells might be involved in causing the observed cell damage during HFRS/HPS. Interestingly, the levels of granzyme B and LDH did not correlate. This is in line with the proposed functions of perforin and granzyme B during the killing of target cells by cytotoxic cells: although granzyme B induces the apoptosis, perforin is needed for granzyme B to enter the cell (18).

The levels of perforin and caspase-cleaved CK18 did not correlate significantly, and although it could be speculated that the increased vascular permeability observed during HFRS/HPS is due to apoptosis caused by hantavirus-specific CD8$^+$ T cells, this remains to be clearly shown. Apoptosis of other epithelial cells might also contribute to the increased levels of caspase-cleaved CK18. The interaction between the virus and the receptor $\alpha_v\beta_3$ integrin, tumor necrosis factor, and/or reactive oxygen species might also induce permeability of endothelial cells (4, 6, 7, 14, 20, 27). The levels of perforin and granzyme B observed during the convalescent phase is similar to those previously reported for healthy individuals (15, 32), but the levels of caspase-cleaved CK18 were clearly higher, indicating that epithelial cell apoptosis might be increased for a prolonged time after infection.

We have shown that elevated levels of extracellular perforin, granzyme B, and epithelial cell apoptosis are induced during acute hantavirus infection. The capillary leakage during HFRS/HPS might be due to apoptosis, and the strong hantavirus-specific CD8$^+$ T-cell responses observed might be responsible for the damage.

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