Clinicopathological Features of Gastric Cancer Cases and Aberrantly Expressed Recoverin

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Cancer-associated retinopathy (CAR) is a paraneoplastic syndrome whereby immunological reactions toward recoverin, a retina-specific calcium binding protein, and other retinal antigens aberrantly expressed in tumor cells lead to the degeneration of retinal photoreceptor cells. Recently we reported that aberrant expression of recoverin was identified in more than 50% of tumor cells and their cell lines from several kinds of cancers, including gastric cancer, lung carcinoma, and other cancers. To elucidate the clinicopathological significance of aberrantly expressed recoverin in cancer cells, we performed immunocytochemical analysis using a monoclonal antibody against human recoverin. Within 18 patients with different clinical stages (I-IV) of gastric cancer, the aberrant expression of recoverin in tumor cells was recognized in 6 out of 18 patients (2 out of 2 stage IA, 1 out of 1 stage IB, 2 out of 3 stage II, 0 out of 7 stage IIIA, 0 out of 1 stage IIIB, and 1 out 4 stage IV). The present data are consistent in part with the previous observations that recoverin-expressing cancer cells induced tumor immunity and provide a favorable prognosis for primary cancer in CAR patients.

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binding protein, and other retinal antigens (Ohguro and Nakazawa 2003). Recoverin is known to play an important role in the light and dark adaptation by regulating rhodopsin phosphorylation and dephosphorylation in a Ca\(^{2+}\)-dependent manner (Kawamura 1991; Ohguro et al. 1996). As a mechanism for the apoptotic cell death of photoreceptor cells observed in CAR, it has been postulated that anti-recoverin antibody generated by unknown mechanisms in some cancer patients penetrates into the photoreceptor cells via the peripheral circulation and causes misregulation of the phototransduction pathway by blocking recoverin function (Adamus et al. 1998; Maeda et al. 2001b).

In the initial step of CAR pathogenesis, several studies suggested that aberrant expression of retina-specific recoverin, serving as a shared antigen between tumor cells and retina, was critical since such aberrant expression of recoverin had been identified in tumor cells in CAR patients but not in non-CAR cancerous patients (Polans et al. 1995; Matsubara et al. 1996; Yamaji et al. 1996). However, in contrast, we have recently found that aberrant expression of retina-specific recoverin occurred in more than 50% of several kinds of cancer cells, and their cell lines obtained from non-CAR cancerous patients (Maeda et al. 2000; Maeda et al. 2001a). Furthermore, we have made the following experimental observations: 1) immunofluorescence labeling by affinity purified recoverin antibody revealed the immunoreactivity toward recoverin as a granular pattern within the cancer cells (Maeda et al. 2001b; Miyagawa et al. 2003); 2) transfection with human recoverin cDNA of A549 cells from human lung adenocarcinoma, which did not express recoverin, exhibited a significant reduction in cell proliferation (Maeda et al. 2000); 3) significant changes were observed in protein phosphorylation patterns in cytosol of A549 cells upon addition of purified recoverin in a calcium-dependent manner (Maeda et al. 2001b) recoverin-specific cytotoxic T lymphocytes (CTL) exist in the peripheral circulation of CAR patients, and these CTL can recognize tumor-expressing recoverin (Maeda et al. 2001a).

These observations correspond well with the favorable prognosis characteristic of malignant tumors underlying CAR. Therefore, it is reasonable to think that aberrantly expressed recoverin may play significant roles in the regulation of cell proliferation of the tumors.

In the present study, to elucidate the clinical significance of aberrant expression of recoverin in patients with malignancies, the presence of recoverin in tumors was compared with clinical factors, including clinical stages, presence or absence of metastasis and prognosis. For this purpose, gastric cancer was studied since it is one of the commonest malignancies in Japan and its clinical and pathological aspects have been most extensively studied. Therefore, the data obtained herein will provide a novel insight into the abenently expressed recover in other malignancies.

**Patients and Methods**

The studies were performed in accordance with our institution’s guidelines and the Declaration of Helsinki on Biomedical Research Involving Human Subjects and protocols were approved by the institution’s Committee for the Protection of Human Subjects. After immunization of mice with human recoverin, prepared using a pET-3d vector inserted human recoverin generously provided from Prof. Satoru Kawamura (Osaka University, Osaka), anti-human recoverin monoclonal antibody was raised by fusion of the immune spleen cells harvested from mice with highest titiers to human recoverin and screening the antibody-producing cells to get stable hybridoma clones. Anti-human recoverin monoclonal IgG was purified by protein G Sepharose column chromatography before use (Miyagawa et al. 2003). The Kato III originated from gastric cancer purchased from American Type Culture Collection was basically established as described previously (Maeda et al. 2000) and was maintained in RPMI 1640 containing 10% fetal bovine serum and antibiotics.
Patients

Gastric carcinoma tissues were obtained from 18 patients who underwent surgery at Hirosaki University Hospital. No retinopathy has been associated with these patients. The definitions of stage grouping were made according to the 13th edition of the Japanese classification of Gastric Carcinoma (Japanese Gastric Cancer Association 1999).

Western blot

Cancer cell line (Kato III) was solubilized in 0.5% Tween 20 in PBS and subjected to Western blot analysis as described previously (Ohguro et al. 1993).

Immunocytochemistry

Tumors, which had been surgically removed, were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.4), embedded in paraffin and sectioned at 3-µm thickness, mounted on subbed slides, and dried. The sections were incubated with anti-human recoverin monoclonal IgG at 1:100 in PBS for 1 hour and rinsed with PBS three times for 5 minutes each. Then the sections were incubated with goat anti-human IgG labeled with FITC (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1:500 in PBS containing 0.035% Tween 20 (T-PBS) at room temperature for 1 hour. The sections were then rinsed three times with PBS for 5 minutes, and mounted on a glass slide using Vectashield fluorescence mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA).

Results

In the present study, we studied aberrant expression of recoverin, a major CAR antigen in tumor tissues obtained from 18 surgically treated patients with several clinical stages of gastric cancer using monoclonal antibody to human recoverin (anti-human recoverin mAb). In western blot analysis, a 26kDa prominent band was recognized by this antibody in cell lysate from Kato III cell lines established from gastric cancer (Fig. 1). Immunofluorescence labeling identified aberrant expression of recoverin in a total of 6 patients among these 18 patients’ specimens (Representative positive and negative of the immunofluorescence labeling in Table 1 are shown in Fig. 2). In comparison to their clinicopathological features, the recoverin expression was more often evident in tumor specimens obtained from patients with early clinical stages (I and II) of gastric cancer than in specimens from advanced stages (III and IV) of this cancer (p=0.0011, Chi-square test), and was more often recognized in tubular carcinoma (4 out of 9) and mucinous...
Adenocarcinoma (1 out of 2) than poorly differentiated carcinoma (1 out of 7). Six years after the surgical operations, 3 out of 4 and 6 out of 11 patients with recoverin positive and negative tumors, respectively, were still alive.

**DISCUSSIONS**

It was identified that recoverin is aberrantly expressed in the cancer cells or their cell lines obtained from CAR patients, and this may trigger the autoimmune reaction causing progressive retinal degeneration (Ohguro and Nakazawa 2003). Preliminary studies had revealed that such aberrant expression of retinal specific recoverin is not identified in cancer cells without retinopathy. These observations suggested that aberrant expression of recoverin in cancer cells is an initial and critical step in the cause of retinopathy. However, in contrast, we found aberrant expression of recoverin in approximately 50% of cancer tissues from non-CAR cancerous patients. This high rate of recoverin expression, which is beyond the incidence rate of retinopathy, allowed us to speculate that this aberrant expression must have additional underlying roles that have been heretofore unknown. To elucidate functional roles of the aberrant expression of recoverin in cancer cells, we transfected recoverin cDNA in A549 cells, which is a lung adenocarcinoma cell line not originally containing recoverin expression, and found that this caused their proliferation to slow down (Maeda et al. 2000). Furthermore, we also found...
that recoverin-specific cytotoxic T lymphocytes (CTL) exist in the peripheral circulation of CAR patients, and these CTL can recognize tumor-expressing recoverin (Maeda et al. 2001a). These observations correspond well with the favorable prognosis characteristic of malignant tumors underlying CAR. Therefore, it is reasonable to theorize that aberrantly expressed recoverin may play significant roles in the regulation of cell proliferation of the tumors. So far, however, no study has been available that examines the relationship between recoverin expression and tumor prognosis. Here, to elucidate the clinicopathological significance of the recoverin expression in malignant tumor, we studied recoverin expression in tumor tissues obtained from 18 surgically treated patients with several clinical stages of gastric cancer, and found aberrant expression of recoverin in a total of 6 patients within this group (Fig. 1 and Table 1). This rate (33.3%) of recoverin expression in gastric carcinoma is less than that (50%) recognized in several tumor cell lines. We assumed...
that the rate of former should be more clinically relevant than the latter because natures of tumor cell lines may be modified from the nature of the original tumors during their cell culture steps. Clinicopathologically, recoverin expression was more often evident in tumor specimens obtained from patients with earlier clinical stages (I and II) and in tumors of lower malignancy pathology type, tubular carcinoma type of gastric cancer.

The mechanisms responsible for the aberrant expression of recoverin in cancer cells are still unknown at present. However, functional roles of recoverin, regulation of rhodopsin phosphorylation (Ohguro et al. 1996) in a calcium dependent manner in photoreceptor cells, allowed us to speculate that recoverin may effect calcium-dependent protein phosphorylation of some G-protein coupled receptors by their specific kinases, GRK (G-protein coupled kinase) homologous to RK in cancer cells. In fact, we have recently found that recoverin was coupled with caveolin-1 and GRK 2, 5 and 6 within tumor cells, and suggested that recoverin may be linked with caveolin-1 dependent regulations, such as tumor progression, metastasis and drug resistance (Miyagawa 2003).

Considering the specific binding of caveolin and G proteins, GRK and other proteins, the association of tumor-expressed recoverin with caveolin-1 and GRKs observed in our present study allowed us to speculate that recoverin may have important functional roles within tumor cells. In terms of pathological roles of caveolin in cancer, Yang et al. (1998) reported that caveolin-1 gene was identified as a metastasis-related gene in the mouse prostate cancer model using differential display-polymerase chain reaction method. It was also reported that elevated expressions of caveolin-1 are recognized in specimens of human patients with prostate and breast cancer (Lavie et al. 1998). Thus, caveolin-1 is suggested to be a novel prognostic marker for clinically confined human prostate cancer. Furthermore, recent studies demonstrated a potential role of caveolin-1 in the resistance of various malignant tumors against multiple neoplastic agents (Lavie et al. 1998; Yang et al. 1998). Although recoverin function in tumor cells is unknown at present, recoverin may potentially be linked with caveolin dependent regulations of tumor progression, metastasis and drug resistance with GRKs. Therefore, to better understand the clinicopathological roles of aberrant expression of recoverin in malignant tumors, further investigations, such as identification of what G-protein coupled receptors are linked with recoverin and GRKs will be next our project.

References
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