Supplementary Data (NDTPLUS-00264-2009)

Methods

Processing of Biopsy Samples and Immunohistochemistry

This study was approved by the Ethics Committee for Human Research of the Faculty of Medicine, Nagoya University (approval number 299, Nagoya, Japan), and the patients provided informed consent prior to participation in the study. Samples of parietal peritoneum were biopsied at operation of gastrectomy. The tissue samples were fixed with 10% buffered formalin overnight, routinely processed for light microscopy and embedded in paraffin. Four-µm sections were cut and stained with hematoxylin and eosin (HE) and Masson’s-trichrome. Part of the tissues were embedded in OCT compound (Sakura Fine Technical, Tokyo, Japan) and frozen in liquid nitrogen, then stored at -80°C for immunohistochemistry. Immunostaining was performed on frozen tissues as described previously (1, 2). Briefly, 4-µm sections were cut with a cryostat, air-dried, and fixed in acetone at room temperature for 10 minutes. Endogenous peroxidase activity was inhibited using 0.1% NaN₃ and 0.3% hydrogen peroxide in phosphate buffered saline (PBS), and nonspecific protein-binding sites were blocked with normal goat serum (Dako, Glostrup, Denmark). Sections were then incubated with primary murine monoclonal antibodies against CD31 (JC/70A; Dako), PAL-E (Abcam, Cambridge, UK), CD68 (EBM11; Dako), mast cell chymase (Millipore, Temecula, CA, USA), MC tryptase (AA1; Dako), mesothelial cell (HBMR-1; Dako) overnight at 4°C. After washing with PBS, sections were treated with a conjugate of polyclonal goat anti-mouse IgG antibodies, and horseradish peroxidase-labeled polymer (Histofine Simple Stain; Nichirei, Tokyo, Japan) as secondary reagent. Enzyme activity was detected by 3-amino-9-ethyl-carbazole (Dako).
Morphological Analysis

CD31-positive vessels, PAL-E positive vessels, CD68-positive macrophages, chymase-positive mast cells and tryptase-positive mast cells were identified and counted using Zeiss Z1 image microscopy and Axiovision Windows software version 4.4 (Carl Zeiss, Oberkochen, Germany). Tissue samples were observed under a microscope at ×200 magnification and at 20 fields (750 × 500-µm) in the submesothelial area, and were assessed using methods of Williams JD for blood vessels, and methods of Jiménez-Heffernan JA and Del Peso G for inflammatory cells (3, 4, 5). The density of blood vessels and inflammatory cells was calculated and was expressed as number per surface length (/mm) or per square millimeter (/mm²), respectively.

Results

We evaluated the expression of inflammatory cells (macrophages and mast cells) and blood vessel density (CD31 and PAL-E) in the peritoneum. We analyzed blood vessel density using two monoclonal antibodies against CD31 and PAL-E, which is considered to be a more specific maker for blood vessels (6). The density of blood vessels was not increased (CD31: 5.60±3.48/mm, PAL-E: 5.69±3.99/mm, mean±SD) as compared with previously reported control tissues (3). We did not identify the obliterated blood vessels in the sections stained with Hematoxylin and Eosin (HE) and Masson’s-trichrome. Infiltration of chymase-positive mast cells (37.57±40.43/mm²) and tryptase-positive mast cells (69.57±33.96/mm²) was predominant in the peritoneum. In contrast, expression of macrophages (7.60±11.22/mm²) was apparently less than that of mast cells.
Reference


