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Co-expression of Thymidine Phosphorylase and Heme Oxygenase-1 in Macrophages in Human Malignant Vertical Growth Melanomas

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Expression of thymidine phosphorylase (TP) is often associated with tumor angiogenesis and/or prognosis in patients. Further, infiltration of macrophages is closely correlated with the depth of tumor and angiogenesis in melanomas. In this study, we examined the expression of TP and an activated macrophage-specific enzyme, heme oxygenase-1 (HO-1), involved in malignancy in 22 cases with melanomas. TP was strongly expressed not only in CD68-positive macrophages in and around tumors, but also in S100 protein-positive melanoma cells, fibroblasts and keratinocytes. By contrast, HO-1 was specifically expressed in macrophages, but only slightly in melanoma cells and other cell types in the stroma of melanomas. We thus observed apparent co-expression of TP and HO-1 in macrophages infiltrating in the late stage of malignant melanomas. There appeared increasing numbers of TP-positive cells in Clark level IV and V melanoma compared with Clark level I (*in situ*) melanoma, and there was also a close correlation between numbers of TP-positive cells and HO-1-positive cells. Both TP- and HO-1-positive macrophages could be observed in the stroma in and around tumors in vertical growth melanomas.

Key words: Malignant melanoma — Angiogenic macrophage — Angiogenesis — HO-1 — TP

The interaction of the stroma with malignant cells has a critical role in tumor growth, invasion and angiogenesis.[1)] Tumor-associated macrophages (TAMs) are a major component of the stroma responsible for tumor development and tumor neovascularization.[2, 3)] Recruited TAMs produce various angiogenesis-related factors and proteinases, such as chemoattractive factors, epidermal growth factor, transforming growth factor α, basic fibroblast growth factor, interleukin-8, vascular endothelial growth factor, metalloproteinases and plasminogen activators, which are thought to be involved in angiogenesis *in vitro*, as well as *in vivo*.[2, 4)] However, thymidine phosphorylase (TP)/platelet-derived endothelial cell growth factor[5)] has a key role in tumor malignancy, as well as angiogenesis, in various human solid tumors including breast cancer,[6, 7)] ovarian cancer,[8)] bladder cancer,[9)] ductal adenocarcinoma of pancreas,[10)] renal cell carcinoma,[11)] head and neck cancer[12)] and colorectal carcinoma.[13)] Immunohistochemically, TP expression has been found in macrophages, stromal cells and glial cells of normal tissue,[14)] and TP expression levels in cancer regions are higher than in their surrounding normal tissues in various tumor types. Toi *et al.*[6)] examined the clinical implications of TP expression in TAMs of 229 primary breast carcinomas tissues, and showed that TP-positive monocytic cells imply a markedly worse prognosis in carcinoma than TP-negative monocytic cells.[6)] TP could be thus a useful marker enzyme for TAMs, which are possibly associated with malignancy in breast cancers.[6)]

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We recently demonstrated that macrophage infiltration could be a useful diagnostic marker of the progression of cutaneous malignant melanomas, and also that production of effective angiogenic factors, interleukin-8 and/or vascular endothelial growth factor, is up-regulated in melanoma cells by macrophage-derived cytokines, tumor necrosis factor α and interleukin-1.[15)] We also showed that increased numbers of infiltrating macrophages are closely associated with heme oxygenase-1 (HO-1) expression in malignant human gliomas.[16)] In this study, we investigated if TP is expressed in TAMs, and also if HO-1 is expressed coordinately with TP in TAMs of human melanomas. The possible association of co-expression of HO-1 and TP in macrophages with activated states of TAMs in melanomas is discussed.

MATERIALS AND METHODS

Antibodies Anti-TP monoclonal antibody was kindly provided by Nippon Roche Research Center (Kamakura). Anti-heme-oxygenase-1 polyclonal rabbit antibody was purchased from Stressgen (BC, Canada). Polyclonal rabbit anti-human S-100 protein antibody was purchased from Nichirei (Tokyo). Mouse monoclonal antibody against the macrophage marker CD68, KP-1 was purchased from DAKO (Glostrup, Denmark).

Tissue preparation Formalin-fixed, paraffin-embedded tissue sections of human malignant melanoma were examined in this study. All patients (n=22) were Japanese.

Classification of tumor levels The thickness of primary melanomas was measured vertically from the granular

layer (or top of an ulcerated surface) to the deepest point of tumor invasion.[17] In this study, the cases were classified into three groups: group 1 comprised Clark level I (*in situ*) melanoma, group 2 comprised Clark level II or III melanomas, and group 3 comprised Clark level IV or V tumors.[15]

Immunohistochemistry of human malignant melanomas The sections were stained using alkaline-phosphatase and anti-alkaline-phosphatase techniques that amplify the primary antibody signal. The stain was developed using a new fuchsin substrate kit (Nichirei), yielding an insoluble red reaction product. A hematoxylin nuclear counterstain was also used. Double immunostaining was performed using the avidin-biotin complex technique with rhodamine or fluorescein isothiocyanate (FITC) as a marker. The immunolabeled specimens were observed under a confocal laser scanning microscope (LSM-GM 200, Olympus, Tokyo) equipped with krypton/argon laser sources. To observe singly immunolabeled specimens (either rhodamine or FITC), the wavelength of the excitation laser light was restricted to 488 nm for FITC and 568 nm for rhodamine by switching the diachronic mirrors to avoid any interference. To simultaneously observe doubly immunolabeled specimens (FITC and rhodamine), both 488- and 568-nm laser lights were used for excitation at the same time. In those cases, we carefully verified the absence of interference in advance, especially in the observations of double-positive sites.

Quantification of the areas of HO-1- and TP-positive cells The melanomas were scanned microscopically to identify the area showing the greatest HO-1- and TP-positive cell density. In the majority of cases, many areas of high density of HO-1- and TP-positive cells were seen at the leading edge of the tumors. The numbers of microvessels and macrophages in a microscopic field (400× magnification) were recorded. The three areas with the highest counts of HO-1- and TP-positive cells were evaluated for each tumor.

RESULTS

We first investigated TP expression in human cutaneous malignant melanomas (n=22). Among five cases of group 1 (*in situ*) melanoma, when melanoma cells were in the epidermis (Fig. 1, a and b), TP expression in melanoma cells was positive (Fig. 1c). Other cell types, such as keratinocytes and fibroblasts also expressed TP. In contrast, in the vertical growth melanoma (Fig. 1d) including groups 2 and 3 melanoma, TP expression was positive in the melanoma and stromal cells (Fig. 1e) in all cases. As shown in Fig. 1f, various levels of TP expression were seen in melanoma cells: the expression of TP in the nucleus was much stronger than that in the cytosol. In the stroma, many macrophages were positive for TP immunoreaction (Fig. 1g). TP-positive macrophages were observed mainly around the tumor.

We next determined if HO-1 is specifically expressed in macrophages in melanomas. Macrophages and melanoma cells were respectively recognized by specific immunostaining with antibodies against CD68 (macrophages) and S100 protein (melanoma cells). Double immunostaining for HO-1 (red color) (Fig. 2, a and b) and CD68 (green color) (Fig. 2, c and d) showed almost complete co-staining of HO-1 and CD68 (Fig. 2, e and f) in the melanoma in all 17 other cases of melanoma of groups 2 and 3. However, we did not observe such co-staining as seen in Fig. 2 when double immunostaining for S100 and HO-1 was performed (data not shown). HO-1 thus appeared to be specifically expressed in infiltrating macrophages rather than in melanoma cells.

We examined if HO-1 is expressed coordinately with TP in melanomas. In one case of group 3 melanoma, HO-1-positive cells seemed to be infiltrating macrophages in the melanoma (red-colored cells) (Fig. 3a). By contrast, a population of TP-positive cells (green colored cells) that were apparently larger cells than HO-1-positive cells was found in melanomas (Fig. 3b). Some TP-positive cells were stained with an antibody against melanoma cell-specific S100 protein (data not shown). Double immunostaining with anti-HO-1 and anti-TP antibodies showed that TP was expressed in not only macrophages, but also melanoma cells and other types of stroma cells (Fig. 3c). HO-1 thus appeared to be selectively expressed in TAMs in melanomas.

We examined if the number of TP-positive cells including melanoma cells and macrophages was associated with the depth of tumor in melanomas (n=22). Compared with tumor group 1, a marked increase in TP-positive cells appeared in tumor groups 2 and 3 (Fig. 4a). The mean TP-positive cell count in group 3 was higher than that in group 2. Thus, the increased TP expression in tumor specimens was correlated with the depth of tumors.

We next investigated if HO-1-positive cells are associated with TP-positive cells in melanomas. When we compared the mean HO-1-positive cell count and mean TP-positive cell count in all 22 melanomas, we observed a significant correlation between HO-1-positive cells and TP-positive cells (Fig. 4b).

DISCUSSION

TP expression appears to be a prognostic marker of various solid tumor types, and it is also an angiogenic marker for various solid tumors (see introduction section). In this study, we observed that the number of TP-positive cells was closely associated with the depth of invasion of primary melanomas. TP expression was observed not only in melanoma cells, but also in TAMs and other types of

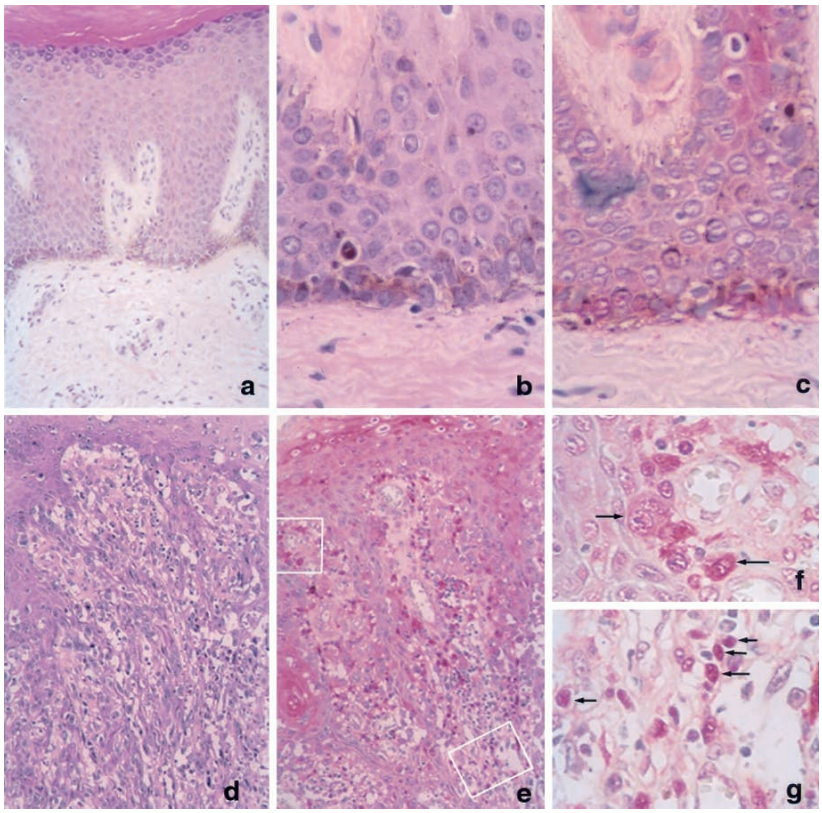

Fig. 1. Immunohistochemical staining of TP in cutaneous malignant melanomas. (a, b) Group 1 (*in situ*) melanoma in hematoxylin-stained tissue section (a, ×100; b, ×400). (c) Tissue section from the periphery of the same tumor, probed with an antibody to the TP antigen (×400). (d) Group 3 melanoma in hematoxylin-stained tissue section (×100). (e) Tissue section from the periphery of the same tumor, probed with an antibody to the TP antigen (×100). (f) Hypermagnification of group 3 melanoma (×400) in the white upper box in Fig. 1e. Arrows show melanoma cells. (g) Hypermagnification of group 3 melanoma (×400) in the lower white box in Fig. 1e. Arrows show macrophages.

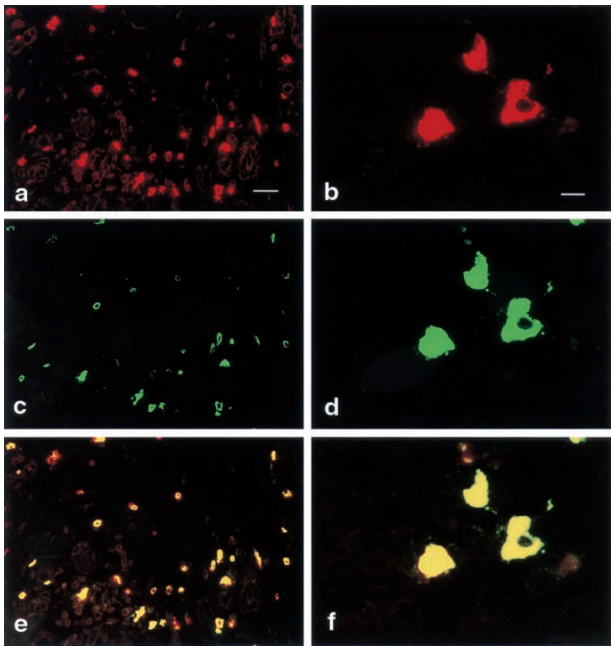

Fig. 2. Double immunostaining for HO-1 and CD68 in group 3 melanoma. Immunostaining for HO-1 (red reaction product) (a, b), immunostaining for CD68 (green reaction product) (c, d). Double immunostaining for HO-1 and CD68 (e, f). CD68-positive macrophages express HO-1 (yellow reaction product). HO-1 was expressed in TAMs in melanomas. Scale bar (a) 10 μm, (b) 1 μm.

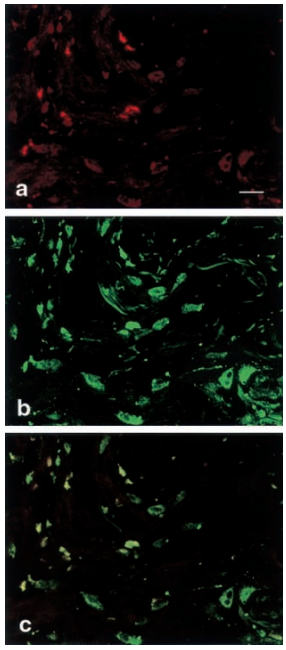

Fig. 3. Double immunostaining for HO-1 and TP in group 3 melanoma. (a) Immunostaining for HO-1. (b) Immunostaining for TP. (c) Double immunostaining for HO-1 (red reaction product), TP (green reaction product). HO-1-positive cells are observed to express TP (yellow reaction product). TP was thus expressed in not only HO-1-positive macrophages, but also other types of cells. Scale bar (a) 10 μm.

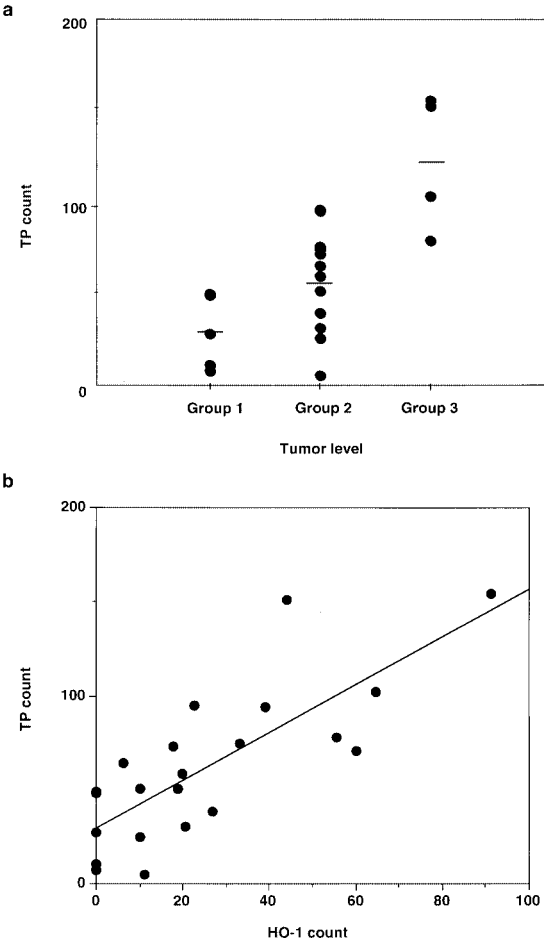

Fig. 4. Association between TP-positive cell count and tumor depth. (a) Group 1 (Clark level I) melanoma, group 2 (Clark level II or III) melanoma; group 3 (Clark level IV or V) melanoma. Mean TP-positive cell count of groups 2 and 3 was significantly higher than that of group 1. (b) Correlation between HO-1-positive cell count and TP-positive cell count (n=22). The regression line y=29.784+1.2654x has a correlation coefficient of r=0.778.

stroma cells in or around melanomas. Furthermore, HO-1 was expressed in CD68-positive TAMs in melanomas, and almost all HO-1-positive macrophages expressed TP in melanomas. There was a marked correlation between HO-1-positive and TP-positive cells, including macrophages and melanoma cells, in all 22 melanomas. The TP expression in TAMs seems specifically correlated with tumor depth in melanoma, rather than TP expression in melanoma cells and other cell types.

Macrophage infiltration in the stroma is often observed in human breast cancer, renal cell carcinomas, gliomas and melanomas.[15, 16, 18, 19] In human gliomas, both immunostaining and *in situ* hybridization assays showed that the HO-1 expression is specific to infiltrating monocytic cells, and TAM infiltrations and the HO-1 expression are closely associated with both malignancy and angiogenesis.[16] Our previous study[15] showed that the number of TAMs and microvessels markedly increased with increasing depth of melanomas. Infiltration of TAMs thus appeared to be closely associated with angiogenesis, as well as malignancy.

Most HO-1-positive TAMs appeared to be positive for TP in melanomas. A mechanism by which the expression of both genes is controlled under the same stimuli could possibly underlie the co-expression of HO-1 and TP in macrophages. Expression of the *TP* gene is highly susceptible to various cytokines including tumor necrosis factor α and interferon α and γ, pH and oxygen stress.[20–22] Induction of the *HO-1* gene was originally considered to be a kind of self-defense mechanism against cytotoxicity of free radicals.[23] Expression of the *HO-1* gene is highly susceptible to various environmental stimuli, such as hypoxia, heavy metals, heat shock and ultraviolet light irradiation.[24, 25] Of various environmental stimuli that modify *HO-1* gene expression, the alteration of oxygen stress or cytokines in tumor stromas might result in enhanced expression of both HO-1 and TP in activated macrophages. We propose that these activated macrophages, related to tumor malignancy and tumor angiogenesis, should be called "angiogenic macrophages." Determination of angiogenic macrophages in tumor stroma could provide a definitive marker for malignancy and angiogenesis in melanomas and other tumors.

In conclusion, infiltration of macrophages expressing HO-1 and TP was found to be closely related with depth of tumor or angiogenic state in human melanomas.

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