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Rapid Communication

Expression of Monocyte Chemotactic Protein-1 in Human Melanoma *In Vivo*

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A common feature of human melanoma is infiltration by monocytes at early stages of tumorigenesis. This infiltration may be highly significant since macrophages have the capacity to alter the behavior of tumor cells. The authors previously demonstrated that the predominant monocyte chemoattractant produced by tumor cells* in vitro *was monocyte chemotactic protein-1 (MCP-1). The authors identify the expression of MCP-1 in pathologic specimens of both primary and metastatic human melanoma but not in normal skin. The finding that MCP-1 is produced by malignant melanoma suggests that specific genes are expressed in tumor cells that can induce the recruitment of monocytes* in vivo. *(Am J Pathol 1992, 140:9–14)

Malignant tumors are frequently characterized by inflammatory infiltrates that are mainly composed of lymphocytes and monocytes/macrophages.[1–3] The macrophage content of tumors is sustained primarily through the recruitment of circulating monocytes, which undergo maturation *in situ* to macrophages.[4] Malignant melanomas serve as an excellent model to study tumor progression since well-characterized melanomas are available for study at each stage of tumorigenesis.[5] Primary cutaneous malignant melanomas in humans are specifically associated with infiltration of monocytes/macrophages.[6–8] In contrast, normal skin and benign melanocytic lesions exhibit little or no monocyte/macrophage infiltration. The presence of monocytes/macrophages at an early stage of melanoma development may be highly significant since proteases and oxygen radicals produced by these cells may facilitate tumor invasion into the underlying connective tissue.[9] Similarly, angiogenic factors produced by macrophages may assist in the formation of a vascular bed to support tumor growth.[10,11] This presence could account for the observation that melanomas infiltrated with mature macrophages may be more aggressive and have a poorer prognosis than those with a small macrophage infiltrate.[12] Paradoxically, macrophages have also been shown to exert tumoricidal effects.[13–15] Since monocytes/macrophages may positively or negatively effect tumor growth and metastasis, their presence is likely to be highly significant.

Studies with nude mice indicate that the degree of monocyte/macrophage infiltration of melanoma is correlated with the amount of monocyte chemotactic activity produced *in vitro*.[16] We previously demonstrated that monocyte chemotactic protein-1 (MCP-1) accounts for virtually all of the monocyte chemotactic activity produced by tumor cells *in vitro*.[17] Other investigators have also detected constitutive MCP-1 expression by tumor cells *in vitro*.[18,19] Studies described here were undertaken to determine whether MCP-1 is expressed in human malignant melanoma *in vivo*. Our results show that MCP-1 expression occurs at early stages of melanoma tumorigenesis and is maintained in metastatic lesions.

Materials and Methods

Immunohistochemistry

Human melanoma and normal skin biopsies were snap-frozen. Tissue sections were fixed by immersion in cold 4% paraformaldehyde after cryostat sectioning. Five pri-

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mary melanomas, five metastatic melanomas, and five normal skin specimens were included for study. MCP-1 expression was examined by both immunohistochemistry and by *in situ* hybridization. For immunohistochemistry, fixed tissue sections were incubated with MCP-1 antiserum (1:500). Rabbit MCP-1 specific antisera raised against purified baboon smooth muscle cell-derived MCP-1 was generously donated by Dr. A. Valente and has previously been described.[17,20] For the negative control, MCP-1 antiserum was pre-incubated with excess antigen, purified MCP-1 (2.5 ug of MCP-1 per μL of MCP-1 antiserum). Experiments with the MCP-1 antiserum and the negative control were carried out simultaneously on different serial sections of the same specimen. Antibodies were localized by an indirect immunoperoxidase technique (avidin-biotin-peroxidase complex) employing diaminobenzidine as a chromogen (Vector Labs, Burlingame, CA). Cells were counterstained with hematoxylin.

In Situ *Hybridization*

The *in situ* hybridization protocol reported by Hoeffler et al. was followed,[21] with modifications that we have previously described.[22] A full length human MCP-1 cDNA probe subcloned in a Bluescript SK vector (Stratagene) was generously provided by Dr. E. Apella.[18] The complementary, antisense MCP-1 riboprobe and control, noncomplementary sense riboprobe were labelled with ^{35}S during *in vitro* transcription. The labelled riboprobes migrated as a single band when analyzed by electrophoresis on 2% agarose/formaldehyde gels. The antisense and sense riboprobes were incubated with tissue sections (300,000 cpm per section) and rinsed as we have previously described.[22] Experiments with the MCP-1 antisense and the negative control sense ribprobes were carried out simultaneously. To positively identify melanoma cells expressing MCP-1 RNA, immunohistochemistry was combined with *in situ* hybridization after the protocol we have described.[22] Briefly, tissue sections were first immunostained with the HMB-45 melanoma specific antibody, and then processed for *in situ* hybridization using the MCP-1 specific complementary or noncomplementary ^{35}S-labelled riboprobe. Cells were counterstained with hematoxylin.

Results

Previous studies have shown that melanoma cells *in vitro* produce monocyte chemoattractants.[16,17,23] Figure 1

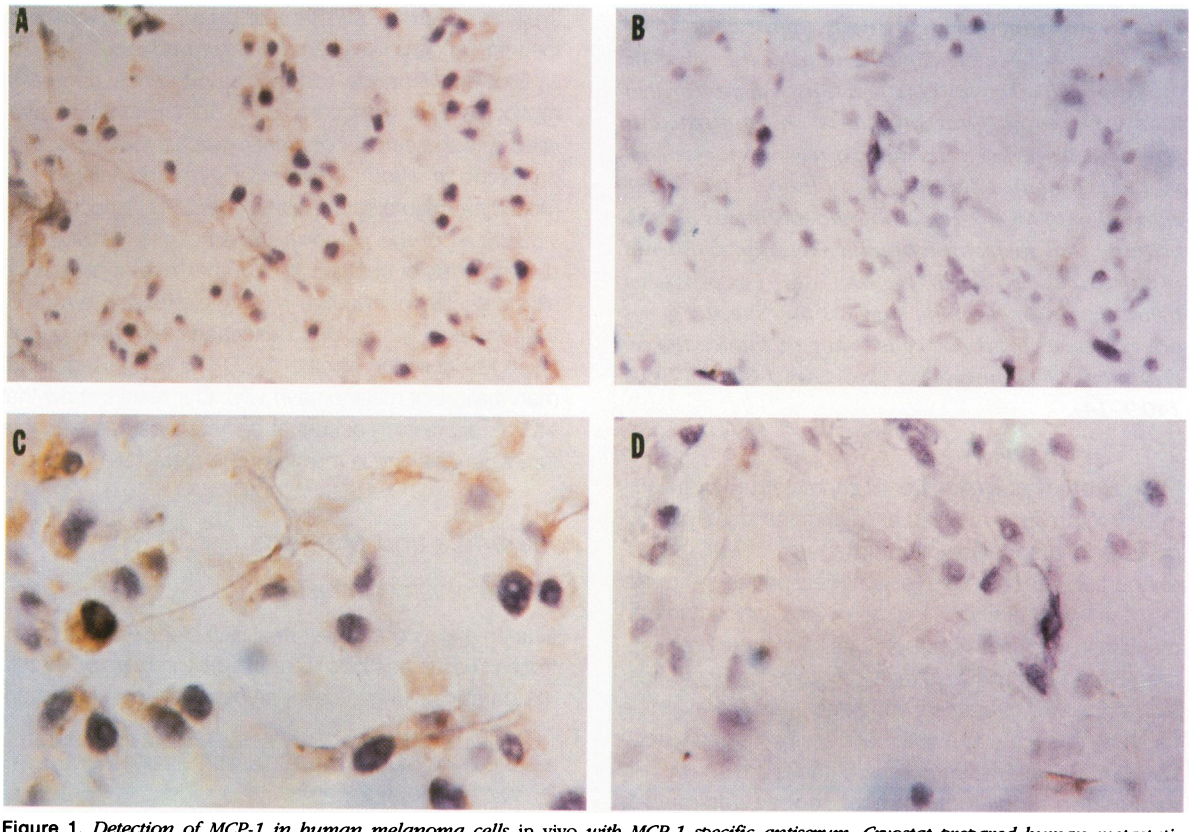

Figure 1. *Detection of MCP-1 in human melanoma cells* in vivo *with MCP-1 specific antiserum. Cryostat prepared human metastatic melanoma tissue sections were incubated with MCP-1 antisera* (**A,C**); *or control, MCP-1 antiserum plus excess purified MCP-1* (**B,D**). *Cells were counterstained with hematoxylin. Original magnification,* **A,B**, ×*400*; **C,D**, ×*630. The pattern of MCP-1 expression shown is representative of the specimen.*

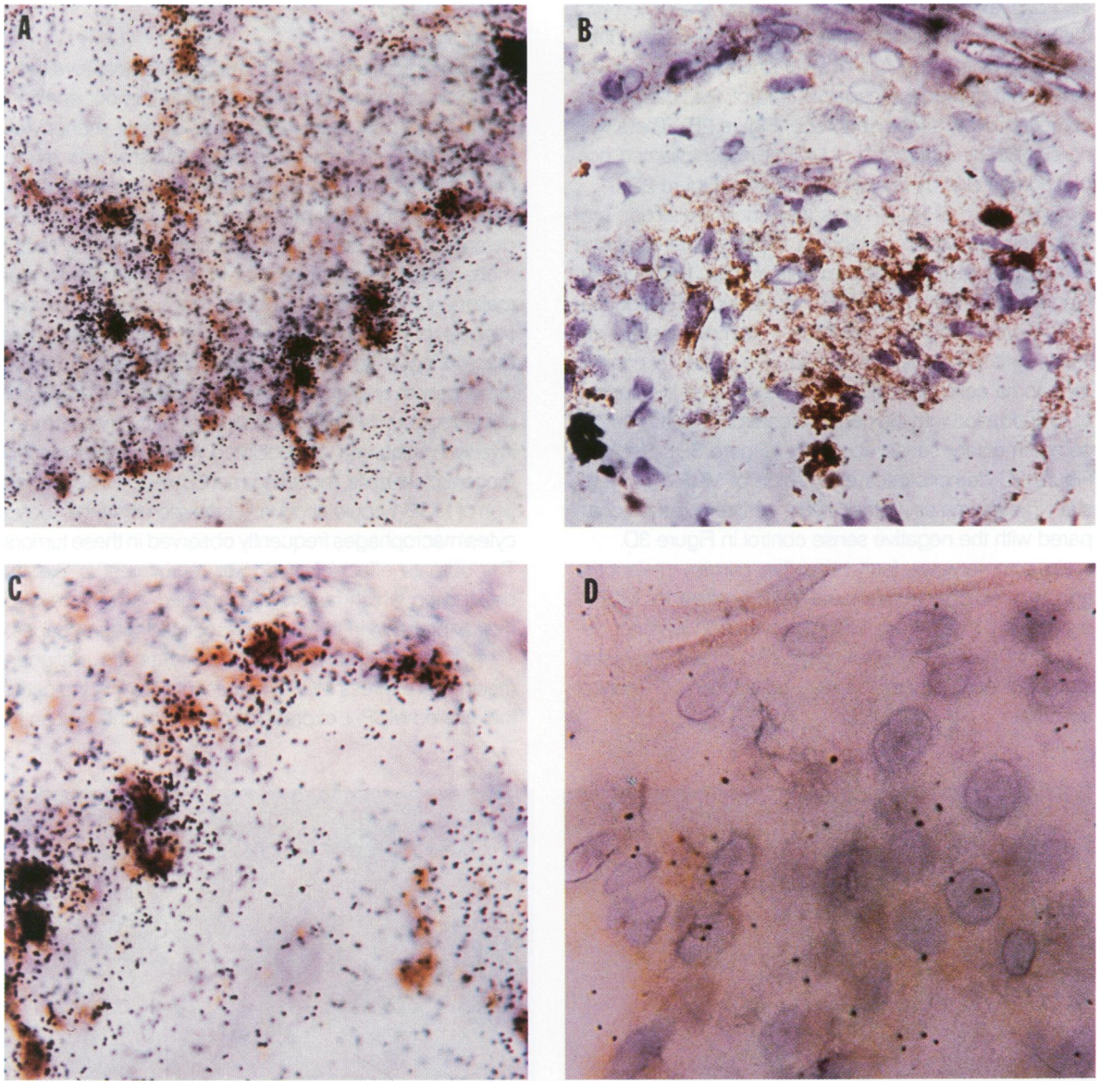

Figure 2. *Detection of MCP-1 mRNA in primary human malignant melanoma of the skin by* in situ *hybridization. Focal expression of MCP-1 in a primary human malignant melanoma (invasive to a measured depth of 0.45 mm) was determined by hybridization with a ^{35}S-labeled complementary "antisense" MCP-1 riboprobe* (A,C) *and lack of specific hybridization with the ^{35}S-labeled noncomplementary "sense" MCP-1 riboprobe* (B). *Immunohistochemistry was combined with* in situ *hybridization on the same tissue section to identify melanoma cells with the HMB-45 melanoma-specific antibody. MCP-1 expression is identified in HMB-45 immunostained melanoma cells* (A,C). *In comparison, there is almost no hybridization of the ^{35}S-labeled complementary antisense MCP-1 riboprobe with normal skin* (D). *Cells were counterstained with hematoxylin. Original magnification,* A,B, ×630; C,D, ×1000. *The pattern of MCP-1 expression shown is representative of the specimen.*

demonstrates the expression of MCP-1 protein in human metastatic melanoma *in vivo* by immunohistochemistry. Immunostaining of the cytoplasm is readily apparent in sections incubated with MCP-1 specific antisera. Immunostaining is not present in the negative control (MCP-1 antibody plus excess purified MCP-1), demonstrating specificity. MCP-1 expression was not detected in four specimens of normal human skin and equivocal expression was noted in a fifth specimen (data not shown). Similarly, we have not detected MCP-1 in the epithelium of noninflamed human lungs nor have we detected MCP-1 expression in normal porcine skin (manuscript in preparation).

The expression of MCP-1 was also demonstrated at the RNA level by *in situ* hybridization. In Figure 2, MCP-1 expression is detected by *in situ* hybridization in invasive melanoma (0.45 mm tumor thickness) using the MCP-1 complementary antisense riboprobe. Melanoma cells are positively identified in the same section by immunohistochemistry using the melanoma-specific antibody, HMB-

45. Focal expression of MCP-1 is clearly associated with the HMB-45. Specificity of the MCP-1 ribroprobe is demonstrated by the absence of silver grains associated with melanoma cells in the negative control, the MCP-1 noncomplementary sense riboprobe (Figure 2B). There was no significant expression of MCP-1 in noninflamed normal human skin by *in situ* hybridization (Figure 2D).

Expression of MCP-1 was also shown in lentigo maligna and metastatic melanoma, representing early and late stages of melanoma tumorigenesis, respectively. Figure 3A demonstrates MCP-1 expression in lentigo maligna. Hybridization of the MCP-1 complementary antisense riboprobe is associated with HMB-45 positive melanoma cells. Specificity is demonstrated by the lack of hybridization with the negative control, the MCP-1 noncomplementary sense riboprobe (Figure 3B). Similarly, Figure 3C demonstrates expression of MCP-1 in metastatic melanoma using the MCP-1 antisense probe compared with the negative sense control in Figure 3D.

In summary, we found that five primary melanoma and five malignant melanoma were positive for MCP-1 expression. In contrast, MCP-1 expression was not detected in four control specimens of normal human skin and equivocal expression was noted in a fifth control specimen.

Discussion

The interaction between a tumor and its host involves a complex cascade of events. During tumorigenesis a given tumor may take advantage of host monocytes/macrophages to facilitate growth, invasiveness and/or metastasis.[2,10,11] One explanation may be that, at the earliest stages, some melanoma cells may escape host defenses. Once selected on this basis, they may recruit monocytes/macrophages that could contribute to their malignant potential through the generation of oxygen radicals, proteases, or growth factors. Tumor-associated macrophages, however, also have the potential to inhibit tumor growth or exert tumoricidal effects.[13–15] It has been observed that the progression from early melanoma to invasive melanoma is associated with the recruitment of mononuclear cells, including monocytes.[7,8] The expression of MCP-1 could account for the recruitment of monocytes/macrophages frequently observed in these tumors. The ultimate effect of monocyte recruitment is likely to depend on which factors are induced and the level of their expression in the tumor-associated macrophages.

The finding that primary and metastatic malignant melanoma express MCP-1 represents the first indication that altered MCP-1 expression occurs during human tu-

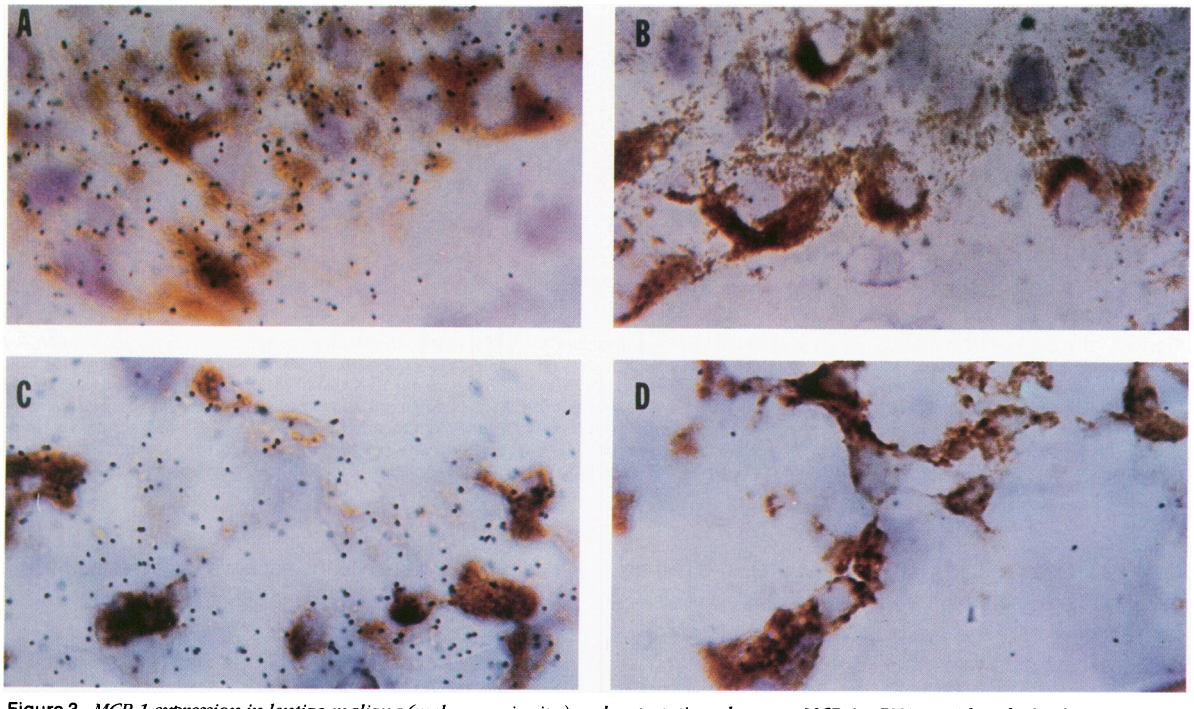

Figure 3. *MCP-1 expression in lentigo maligna (melanoma* in situ*) and metastatic melanoma. MCP-1 mRNA was identified in human lentigo maligna (melanoma* in situ*)* **(A,B)**; *and metastatic melanoma* **(C,D)** *by* in situ *hybridization combined with immunohistochemistry with the HMB-45 antibody to identify melanocytic tumor cells as described in Figure 2. MCP-1 expression was determined by comparing hybridization of the ^{35}S-labeled MCP-1 complementary "antisense" riboprobe* **(A,C)** *versus the ^{35}S-labeled MCP-1 noncomplementary "sense" riboprobe* **(B,D)**. *Cells were counterstained with hematoxylin. Original magnification, ×1000. The pattern of MCP-1 expression shown is representative of both specimens.*

morigenesis. MCP-1 expression by human melanoma cells *in vivo* could account for monocyte recruitment in these tumors. However, the production of MCP-1 alone may not be the sole determinant of monocyte recruitment since other factors, such as inhibitors of monocyte chemotaxis, may also be expressed.[23] MCP-1 expression is also believed to be associated with other processes that have a significant monocyte infiltrate. MCP-1 expression is induced in vascular smooth muscle and endothelial cells by pro-inflammatory cytokines *in vitro,* suggesting a role for MCP-1 in vascular inflammatory processes such as atherosclerosis[24–26] This is supported by *in vivo* evidence that MCP-1 expression can be detected in atherosclerotic plaques, whereas MCP-1 is not detected in normal blood vessels.[27] Thus, different physiologic or pathophysiologic processes may share a common mechanism for inducing monocyte recruitment.

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