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Expression of acute and late-stage inflammatory antigens, c-fms, CSF-1, and human monocytic serine esterase 1, in tumor-associated macrophages of renal cell carcinomas

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Abstract Purpose: Tumor cells influence the differentiation of infiltrating macrophages. In the present study, the differentiation of macrophages in renal cell carcinomas was investigated with special regard to their possible role in tumor growth and spread. **Methods:** Macrophages were characterized by means of immunohistochemistry of the Ki-M1P, 25F9, MRP8, MRP14, and MRP8/14 antigens and by means of in situ hybridization of CSF-1, its c-*fms*-coded corresponding receptor, and human monocytic serine esterase-1 (HMSE-1) mRNA. Macrophage subgroups were quantified within central tumor tissue, the corresponding tumor host interface, and tumor-free tissue and correlated with tumor necrosis, fibrosis, and tumor stage and grade. **Results:** Macrophage density was much higher within tumor tissue and the tumor/host interface than in tumor-free tissue. Well-differentiated carcinomas showed a lower degree of macrophage density than less-differentiated carcinomas. Tumor-associated macrophages could be divided into an active inflammatory type ($MRP14^+$, $MRP8/14^+$) and into a late-phase inflammatory type ($25F9^+$, $MRP8^+$). Necrosis was seen in less-differentiated carcinomas and associated with a significantly increased density of $MRP14^+$ macrophages, which, however, did not correlate with the extent of necrosis. The density of $25F9^+$ macrophages was correlated with an extensive connective tissue formation and an advanced tumor stage. c-fms, CSF-1, and HMSE-1 mRNA expression did not discriminate between the macrophage subgroups. **Conclusions:** Tumor-associated macrophages of the late-stage inflammatory type potentially support the spread of renal cell cancer. CSF-1 derived from tumor cells and macrophages acts as a monocyte attractant and induces macrophage differentiation able to modulate the extracellular matrix rather than to exert cytotoxicity.

Key words Tumor-associated macrophages · Renal cell cancer · Macrophage differentiation

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Introduction

Macrophage functions depend on their maturation and differentiation, which are influenced by cytokines and components of the extracellular matrix. Tumor-associated macrophages either take a direct part in the antitumor response by exerting antibody-dependent cytotoxicity and by producing cytotoxic substances, such as aggressive reactive oxygen intermediates, nitric oxide, and tumor necrosis factor α (TNFα), or indirectly by presenting tumor antigens to lymphocytes. In vitro a tumoricidal macrophage phenotype can be primed by stimulation with macrophage-colony-stimulating factor (CSF-1) [25], granulocyte/macrophage-colony-stimulating factor [15] or interferon γ [1]. However, it can be demonstrated, that tumors are able to escape from the effects of cytotoxic macrophages through the secretion of anti-inflammatory cytokines [6, 7, 24], leading to promotion of tumor growth and spread. Recently, Siegert et al. were able to show a significant reduction of oxygen radical production in macrophages after incubation with cell culture supernatants derived from colon cancer cell lines [29]. In addition, in breast cancer the density of tumor-associated macrophages was significantly correlated with a reduced relapse-free survival [18]. Furthermore, it has been demonstrated that macrophage-derived cytokines such as TNFα, macrophage chemoattractant protein 1, and vascular endothelial growth factor can stimulate angiogenesis [8, 10, 19, 21].

Tumor-associated macrophages can be characterized by activation-associated antigens expressed during the

early or late phase of inflammation. The expression of molecules of the calcium-binding protein family, such as MRP14 and the heterodimer MRP8/14, is associated with acute inflammatory macrophages and activated macrophages during chronic inflammation, respectively, expressing proinflammatory cytokines such as TNFα and oxygen radicals as cytotoxic factors [2, 22, 27]. In contrast, the MRP8 and 25F9 antigens are expressed by macrophages during the late phase of chronic inflammation [9, 33, 34]. In gastric cancer and malignant melanoma the $25F9^+$ macrophage subtype was associated with an advanced tumor stage [3, 13].

Monocytes express low levels of the macrophage growth factor CSF-1 but relatively high levels of the corresponding receptor coded by the protooncogene c-*fms*. In macrophages and giant cells (e.g. in cases of sarcoidosis) secreting high amounts of free oxygen radicals the protooncogene c-*fms* is down-regulated compared to its expression in monocytes, whereas CSF-1 is strongly expressed [16, 17]. Human monocytic serine esterase 1 (HMSE-1) is a lineage-specific esterase of the monocyte/macrophage system [31, 32]. Its expression is low in peripheral blood monocytes but increased in activated macrophages such as alveolar macrophages, macrophages of granulomas, and giant cells [28]. These macrophage differentiation types express MRP14 and MRP8/14 [5]. Therefore, HMSE-1 expression may indicate acute inflammatory macrophages and activated macrophages of chronic inflammation with potential cytotoxic properties. A potential cytotoxic function for HMSE-1 has recently been claimed, because monocyte esterase deficiency could be shown to be associated with an increased incidence of malignant neoplasms in humans [23].

The aim of the present study was to analyze the recruitment and differentiation of tumor-associated macrophages by means of immunohistochemistry of macrophage-activation antigens and by in situ hybridization of c-fms, CSF-1, and HMSE-1 mRNA. We asked, whether a specific differentiation pathway for cytotoxic macrophages could be defined by these analyses.

Table 1 Pathological data of the cases analyzed

Pathological data	*n*
Tumor stage	
Localized (≤pT2, N0, M0)	19
Advanced (pT2, ≥N1 or M1; ≥pT3)	17
Histology	
Clear cell	29
Chromophilic/papillary	3
Chromophobe	1
Sarcomatoid	3
Grade	
I	10
II	20
III	6

Materials and methods

Tumor specimens

Thirty-six nephrectomy specimens from patients with renal cell carcinoma were freshly received from the Urological Department of the University of Göttingen. The patients had not received any therapy prior to tumor resection. The histological typing and grading of renal cell carcinoma was performed according to Thoenes et al. [30]. A synopsis of pathological data is given in Table 1.

Immunohistochemistry

Tissue samples of tumor and tumor-free areas were immediately fixed in 4% neutral buffered formalin and embedded in paraffin. For immunohistological analyses and in situ hybridization, 2-µm-thick serial paraffin sections were mounted on organo-silane-coated slides and air-dried overnight at 40 °C. Immunohistochemistry was performed by the alkaline phosphatase/anti-(alkaline phosphatase) method [4] with New fuchsin as chromogen. All incubations were carried out at room temperature in a humidified chamber. Antibodies were diluted in phosphate-buffered saline (pH 7.4) containing 5% bovine serum albumin. Primary antibodies were incubated for 120 min. The antibodies applied are listed in Table 2.

Non-radioactive in situ hybridization of HMSE-1, c-fms, and CSF-1

Tissue sections were prepared as described above under RNA se-reduced conditions. To abolish RNase activity, glassware was sterilized for 6 h at 180 °C and water was treated with diethylcarbazol. For nonradioactive in situ hybridization a 0.45-kb cDNA fragment of the *HMSE-1* gene, a 1.2-kb cDNA fragment of the c-*fms* gene, and a 0.46-kb cDNA fragment of the *CSF-1* gene were subcloned into the multiple cloning site of the pBluescript II KS+/− phagemid (Stratagene, Calif., USA). Digoxigenin-labeled antisense and sense riboprobes were generated from cDNA templates by in vitro transcription using T3 and T7 polymerase according to the manufacturer's instructions (Roche, Mannheim,

Table 2 Applied antibodies

Antigen	Antibody dilution	Main cellular distribution	Source
Ki-M1P (CD68)	1:1500[a]	Pan-monocyte/macrophage antigen	Own laboratory
MRP14 (Mac 387)	1:50[b]	Macrophages of acute inflammatory phase	Dako, Hamburg, Germany
MRP8/14 (27E10)	1:50[a]	Macrophages of the active phase in chronic inflammation	Dianova, Hamburg, Germany
25F9	1:50	Macrophages of the down-regulated inflammatory phase	Dianova
MRP8	1:50[a]	Macrophages of the late stage of inflammation	Dianova

[a] Microwave pretreatment
[b] Protease pretreatment

Germany). In situ hybridization was performed as previously decribed [28].

Morphometrical analyses

Representative areas of tumor tissue, of the tumor/host interface and of cortical and medullary tumor-free tissues of the same nephrectomy specimen were analyzed. The tumor/host interface was defined as a 500-μm-wide area consisting of tumor tissue and adjacent tumor-free tissue. mRNA expression was analyzed semi-quantitatively. Immunohistologically labeled macrophages were counted in a minimum of 30 microscopic fields, using a standardized ocular optical grid at 400× magnification, and expressed as the number of cells per 0.1 mm^2.

The extent of tumor necrosis and connective tissue formation was analyzed in hematoxylin/eosin-stained tissue sections corresponding to the complete tumor area used for the evaluation of macrophage infiltration. Values were assessed by using a point-sampling ocular optical grid with 24 sampling points (Zeiss, Göttingen, Germany) at 80× magnification.

Statistical analysis

Results were analyzed for statistical significance by the paired Wilcoxon rank-sum test and the *U*-test. For correlation analysis the Spearman rank-correlation test was used.

Results

Quantification and characterization of the macrophage infiltrate

The total macrophage infiltrate was quantified by immunohistochemical evaluation of the CD68 (Ki-M1P) expression. Macrophage density within tumor tissue ranged from 31 macrophages/0.1 mm^2 to 1644 macrophages/0.1 mm^2. Within tumor tissue and the tumor/host interface the macrophage density was higher than that in tumor-free tissue. The macrophage density within the tumor/host interface was higher than that in central tumor tissue.

$MRP14^+$ macrophages, comprising only a subset of the total macrophage infiltrate, were also higher within the tumor/host interface and in tumor tissue than in tumor-free tissue. $MRP14^+$ macrophages amounted to 15%, 11%, and 17% of the total macrophage infiltrate within tumor tissue, the tumor/host interface, and tumor-free tissue respectively.

Immunolabeling of the heterodimer MRP8/14 did not reveal any difference in infiltration between tumor-free tissue, the tumor/host interface or tumor tissue. $MRP8/14^+$ macrophages amounted to 29%, 38%, and 29% of the total macrophage infiltrate within tumor tissue, the tumor/host interface, and tumor-free tissue respectively. Macrophages expressing 25F9 and MRP8 were detected at higher density within tumor tissue and the tumor/host interface than in tumor-free tissue.

Macrophage counts are summarized in Table 3; characteristic macrophage types are shown in Fig. 1.

Macrophage density and tumor necrosis

The extent of necrosis within representative tumor areas was quantified by point sampling and correlated with the macrophage infiltrate in vital tumor tissue. The rims of necrotic foci were characterized by pallisading macrophages. The density of all macrophage subtypes was significantly higher within tumors showing necrosis than in tumors without necrosis. This held specially true for $MRP14^+$ macrophages. However, the infiltrate density of $MRP14^+$ macrophages correlated negatively with the total area of necrosis as measured by point sampling ($r = -0.82$). In Table 4, macrophage density counts with respect to tumor necrosis are summarized.

Macrophage density and tumor fibrosis

Fibrotic and cystic changes are typical features for most renal cell carcinomas. The extent of connective tissue formation was quantified by point sampling. Morphometric analysis revealed one group of tumors with a low degree of hyalinization (up to 14% of the total central tumor area) and a second group with extended hyalinization (up to 47% of the total central tumor area). Characterization of the macrophage infiltrate revealed that a high density of interstitial $25F9^+$ intratumoral macrophages was associated with an extended hyalinization of tumor tissue, whereas the $Ki\text{-}M1P^+$ macrophage infiltrate and the numbers of $MRP8^+$, $MRP8/14^+$, and $MRP14^+$ macrophages were reduced or unaltered (Table 5).

Within cystic regressive changes, macrophages ($Ki\text{-}M1P^+$) differentiated predominantly towards a foam cell phenotype that was also associated with a high expression of the 25F9 antigen but a low expression of antigens associated with acute inflammation (Fig. 1D).

Table 3 Macrophage density in central tumor tissue, the tumor host interface, and tumor-free tissue. All values are expressed as means (positive cells/0.1 mm^2) with standard deviation and median in parentheses. No statistically significant differences were found between central tumor tissue and the tumor/host interface, whereas differences were found in comparison to tumor-free tissue

Location	CD 68*[1]	25F9*[2]	MRP8*[3]	MRP14*[4]	MRP8/14*[5]
Tumor center	375.3 ± 362.5 (293.5)	37.4 ± 56.4 (13)	14.3 ± 16.1 (8.5)	56.2 ± 60.5 (30.5)	17 ± 27.8 (3.5)
Tumor host interface	370 ± 231 (359)	26.6 ± 32.1 (14)	10.8 ± 7.6 (8)	42.4 ± 35.2 (33)	10.8 ± 13.7 (5)
Tumor-free tissue	110.9 ± 99.1 (80)	3.5 ± 5.4 (1)	5.1 ± 4.4 (3.5)	19.4 ± 20.1 (13)	8.8 ± 12.6 (3)

*[1–5] Statistical values comparing tumor-free tissue to central tumor tissue: *[1] $P = 0.0007$; *[2] $P = 0.003$; *[3] $P = 0.0052$; *[4] $P = 0.0034$; *[5] $P > 0.05$

A correlation could not be found between the density of macrophages and macrophage subgroups and the tumor infiltration patterns, such as diffuse infiltration or induction of a fibrous pseudocapsule at the tumor/host interface as revealed by point-sampling quantification of interstitial connective tissue.

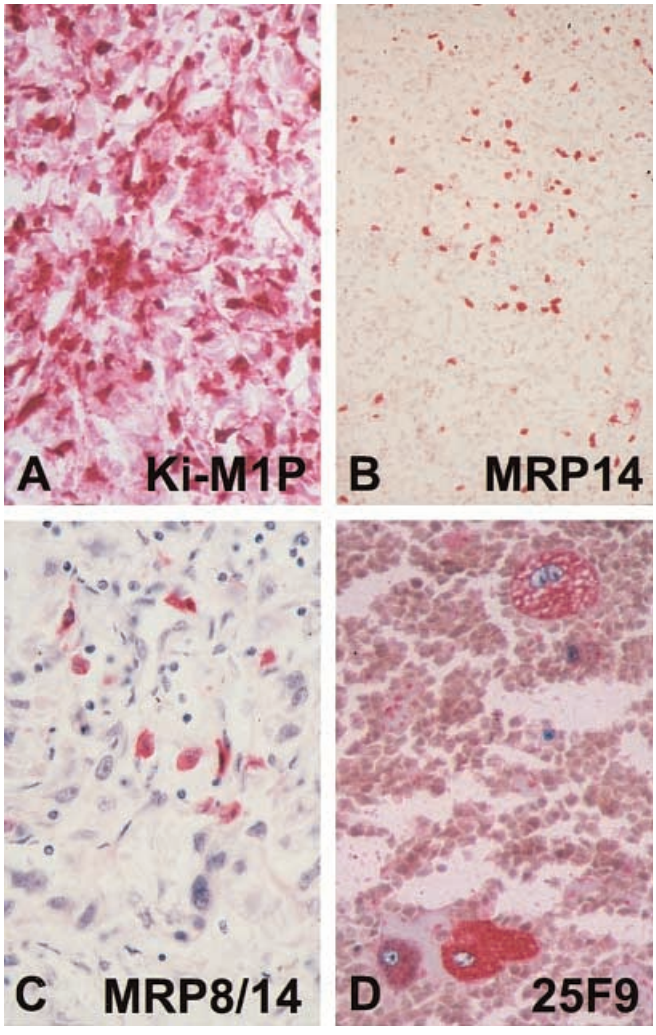

Fig. 1A–D Density and subtypes of tumor-associated macrophages. Tumor-associated macrophages in a grade 3 renal cell carcinoma. **A** Characteristically, a dense infiltrate of Ki-M1P$^+$ macrophages can be observed. In contrast, MRP14$^+$ (**B**) and MRP8/14$^+$ (**C**) macrophages comprise only a subpopulation of the total Ki-M1P$^+$ macrophage infiltrate. MRP8/14$^+$ macrophages show a cytoplasmic but no membraneous antigen expression (**C**) [alkaline phosphatase/anti-(alkaline phosphatase) (APAAP) stain; **A**, **B** original magnification ×200; **C** original magnification ×630]. In renal cell carcinomas regressive changes develop and attract macrophages that develop a large or foamy cytoplasm expressing the late-stage inflammatory antigen 25F9 (**D**; APAAP stain; original magnification ×400)

Macrophage density and tumor stage and grade

The density of 25F9$^+$ interstitial macrophages correlated with an advanced tumor stage at the time of surgical removal, as indicated by vascular invasion, penetration of the organ capsule or metastasis. MRP8$^+$, MRP14$^+$, and MRP8/14$^+$ macrophages did not show any association with the tumor stage (Table 6). Comparison of grade 1 carcinomas with less-differentiated carcinomas (grades 2 and 3) revealed a higher density of Ki-M1P$^+$ macrophages and of MRP14$^+$ macrophages in less-differentiated carcinomas (Table 7).

mRNA expression of HMSE-1, c-fms, and CSF-1

In situ hybridization revealed a significantly higher density of macrophages expressing HMSE-1 mRNA in tumor tissue than in tumor-free tissue ($P < 0.0001$, *U*-test). This also held true for the expression of c-fms mRNA ($P < 0.0001$, *U*-test) and CSF-1 mRNA ($P < 0.05$, *U*-test). In serial sections, comparison with Ki-M1P-immunolabeled macrophages revealed that HMSE-1 mRNA, c-fms, and CSF-1 mRNA was expressed in a broad range of morphologically distinguishable macrophage subtypes such as small macrophages, macrophages with spindle-cell morphology or large cytoplasm, foamy macrophages in pseudocystic regressions, and giant cells of the foreign-body type. Thus, the immunohistochemically determined macrophage subgroups could not be distinguished by differential HMSE-1, c-fms, and CSF-1 mRNA expression. Only the expression of c-fms mRNA was lower in foamy macrophages and mature macrophages expressing the 25F9 antigen (Figs. 1D, 2A–E).

CSF-1 mRNA could also be detected in tumor epithelia of all renal carcinomas (Fig. 2F). In vitro this result could be confirmed by analysis of eight renal cancer cell lines all of which expressed CSF-1 mRNA when the reverse transcriptase polymerase reaction was applied (data not shown).

Discussion

Macrophages comprise a major component of the immune infiltrate in renal cell carcinomas. As observed in

Table 4 Macrophage density and tumor necrosis. All values are expressed as in Tables 3

Tumor type	CD 68*[1]	25F9*[2]	MRP8*[3]	MRP14*[4]	MRP8/14*[5]
Without necrosis	358.6 ± 441.5 (217.5)	20.9 ± 28.7 (12)	10.4 ± 14.5 (3.5)	36.1 ± 47 (20.5)	8.9 ± 18.8 (1.5)
With necrosis	406.2 ± 101.7 (282)	72.9 ± 80.1 (41)	21.3 ± 17.4 (16)	96.7 ± 66.2 (83.5)	37.3 ± 37 (18)

*[1–5] Statistical values comparing tumors with and without necrosis: *[1]$P = 0.024$; *[2]$P = 0.028$; *[3]$P = 0.017$; *[4]$P = 0.002$; *[5]$P > 0.05$

Table 5 Macrophage density and fibrosis. All values are expressed as in Table 3

Tumor type	CD 68**	25F9*	MRP8**	MRP14**	MRP8/14**
Without increased fibrosis	415.4 ± 392.8 (354)	6.9 ± 4.8 (6.3)	71.3 ± 65.3 (47.8)	17.2 ± 26.5 (8.9)	45.3 ± 62.2 (22.2)
With increased fibrosis	196 ± 119.1 (222)	37.6 ± 7.2 (37.1)	23.9 ± 29.3 (11.1)	18.2 ± 31.7 (5.5)	11.8 ± 11.7 (10.6)

*·** Statistical values comparing tumors with low and increased fibrosis: $*P < 0.0001$; $**P > 0.05$

Table 6 Macrophage density and tumor stage. All values are expressed as in Table 3

Tumor stage	CD 68**	25F9*	MRP8**	MRP14**	MRP8/14**
Localized	341.1 ± 389.2 (222)	26.8 ± 60.7 (10)	15.4 ± 19 (9)	54.1 ± 64.8 (24)	12.9 ± 24.5 (1.5)
Advanced	420 ± 334.4 (354)	56.8 ± 51.9 (32)	12 ± 10.7 (8.5)	59.4 ± 56 (43)	17.3 ± 29.6 (7.5)

*·** Statistical values comparing localized and advanced tumors: $*P = 0.0069$; $**P > 0.05$

Table 7 Macrophage density and tumor grade. All values are expressed as in Table 3

Grade	CD 68*	25F9***	MRP8***	MRP14**	MRP8/14***
1	202 ± 180.9 (140)	19.1 ± 19.4 (12.5)	7.8 ± 8 (5)	26.5 ± 32.7 (11)	12.7 ± 23.5 (1.5)
2 and 3	490.8 ± 409.5 (359.5)	50.9 ± 68.6 (18)	18 ± 19 (10)	70.3 ± 67.9 (43)	20.3 ± 31 (7.5)

*·**·*** Statistical values comparing grade 1 and grade 2/3 carcinomas: $*P = 0.004$; $**P = 0.027$; $***P > 0.05$

the present study, the macrophage infiltrate within tumor tissue and the tumor/host interface was significantly higher than in tumor-free tissue. In addition, the macrophage density in well-differentiated carcinomas was lower than in less-differentiated carcinomas. Up to now there have been no investigations into the relation between tumor grade and macrophage recruitment. In a recent study it could be demonstrated that a differential endothelial expression of adhesion molecules, such as VCAM-1, may be involved in the process of macrophage recruitment [14]. In addition, Leek et al. suggested that macrophage recruitment may be caused by chronic hypoxia in rapidly growing tumors, leading to tumor necrosis [20].

In the present study two subgroups of tumor-associated macrophages within renal cell carcinomas could be characterized: one group expressed the antigens 25F9 and MRP8, which occurred in macrophages of the late inflammatory reaction; the second group expressed the antigens MRP8/14 and MRP14 typical for the active inflammatory reaction. As already reported for malignant melanomas [3] and gastric cancer [13], $25F9^+$ macrophages were associated with an advanced stage of renal cell carcinoma. The association between intratumoral connective tissue formation and $25F9^+$ macrophages in renal cell carcinomas is probably an indicator of the potency of this macrophage subtype to induce stroma formation by factors such as transforming growth factor β, platelet-derived growth factor, and vascular endothelial growth factor. This assumption is in line with the findings of Hauptmann et al. who found that $25F9^+$ macrophages were associated with desmoplasia and stimulation of tumor cell proliferation in colon cancer [11, 12].

Tumor cytotoxic properties have so far been mostly investigated under in vitro conditions. An effective antitumor function of macrophages in vivo, however, still remains to be demonstrated, especially in view of the limited success of macrophage-based adoptive immunotherapy strategies [1]. Among the various macrophage subtypes, $MRP14^+$ macrophages especially, as shown in this study, were associated with tumor necrosis. Clear evidence for a macrophage-derived cytotoxic effect, however, could not be established, because the density of $MRP14^+$ macrophages was negatively correlated with the extent of necrosis and not correlated with an organ-confined tumor stage. Furthermore, the low density of $MRP8/14^+$ macrophages able to secrete cytotoxic factors (mainly observed during an active inflammatory reaction) rather indicates a suppressed cytotoxic macrophage response. These observations are in line with in vitro data showing that conditioned medium derived from colon carcinoma cell lines was able to suppress the production of reactive oxygen intermediates even in inflammation-activated macrophages [29]. In addition, we could observe a significantly lower microvessel density in renal cell carcinomas with necrosis than in carcinomas without necrosis (data not shown). Therefore, tumor necroses are more likely induced by local hypoxia than by macrophage-driven cytotoxicity.

In situ hybridization of tumor-associated macrophages revealed that the morphologically and immunhistologically different macrophage subgroups did not express c-fms and CSF-1 mRNA in a different manner. The early and late inflammatory subgroups of macrophages with their different functional properties could not be distinguished on the basis of c-fms and CSF-1 expression. In addition, CSF-1 mRNA could be observed within tumor cells, supporting the hypothesis that macrophage recruitment and differentiation may be stimulated not only in an autocrine but also in a paracrine fashion [17]. CSF-1 expressed in renal carcinoma

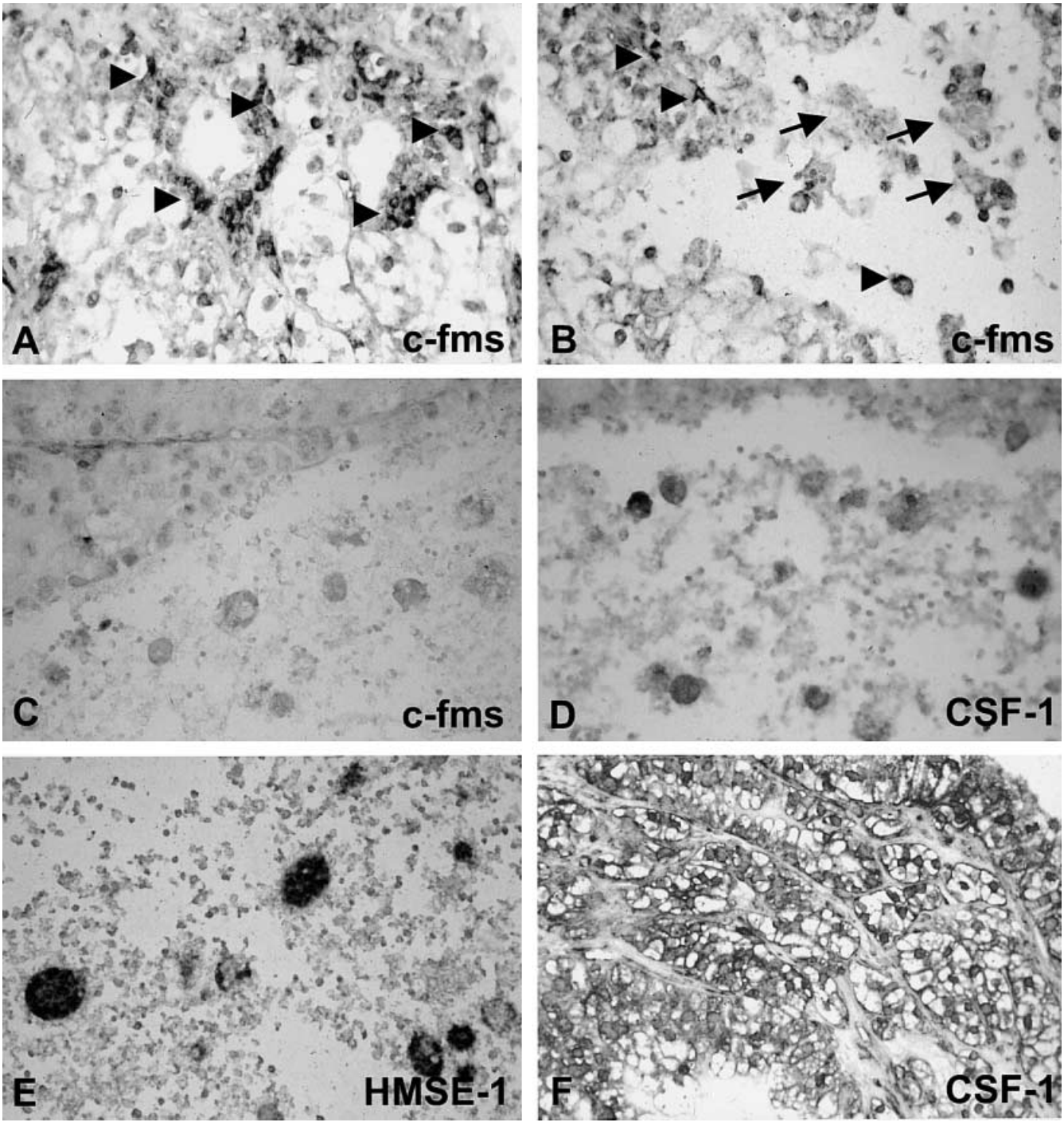

Fig. 2A–F Expression patterns of c-fms, CSF-1, and HMSE-1. **A** Interstitial macrophages (*arrowhead*) express high amounts c-fms mRNA. In contrast, macrophages with a large cytoplasm express low levels of c-fms mRNA (**B**). Foamy macrophages are negative for c-fms mRNA (**C**), but express high levels of CSF-1 (**D**) and HMSE-1 (**E**) (nonradioactive in situ hybridization, original magnification ×400). CSF-1 mRNA is also expressed in tumor cells (**F**); hybridization signals are perinuclear and localized beneath the cytoplasmic membrane because of the glycogen storage of clear cell carcinomas (nonradioactive in situ hybridization, original magnification ×200)

cells has been shown to be one candidate that suppresses the cytotoxic properties of activated macrophages [7]. In a mouse osteopetrosis model, characterized by CSF-1 deficiency, Nowicki et al. found that CSF-1-dependent macrophage differentiation is involved in the formation of tumor stroma such as neoangiogenesis [26]. Thus, it can be assumed that development of $25F9^{+}$ macrophages is dependent on CSF-1 and that they modulate the tumor stroma, favoring tumor progression. This is supported by the finding that tumor cells are also able to suppress the production of reactive oxygen

intermediates in $25F9^+$ macrophages [29]. The increased expression of HMSE-1 mRNA in such tumor-associated $25F9^+$ macrophages does not necessarily reflect an effective cytotoxic potency.

In summary, it could be demonstrated that tumor-associated macrophages in renal cell carcinoma differentiate into an active inflammatory phenotype that is not correlated with an inhibition of tumor spread, and into a late chronic inflammatory phenotype associated with an advanced tumor stage most probably as a result of the secretion of modulating factors by the extracellular matrix.

The attraction and differentiation of such macrophages involved in the formation of tumor stroma rather than in cytotoxic activities seems to be influenced by CSF-1 secreted by the tumor cells and macrophages themselves.

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