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Expression of RCAS1 protein in microglia/macrophages accompanying brain tumours. An immunofluorescence study

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Abstract

The expression of protein RCAS1 (receptor-binding cancer antigen expressed on SiSo cells), possibly involved in the mechanisms of evasion of immune surveillance by tumours, has been studied in brain astrocytomas grade III and IV and in metastatic carcinomas to the brain by means of double immunofluorescence with antibodies against RCAS1 and respectively anti-GFAP (astroglia) or CD68 or CD74 (macrophages/microglia). Expression of RCAS1 has been reported in many types of carcinomas and in some normal cells, including bone marrow macrophages. Nakabayashi and co-workers recently reported expression of RCAS1 in gliomas. So far no attention has been paid to expression of RCAS1 in non-neoplastic cellular elements of tumours such as macrophages and to the expression of RCAS1 in metastatic carcinomas. We found expression of RCAS1 co-localizing with GFAP+ cells of gliomas and with CD68 and CD74 in large macrophages infiltrating metastatic and primary tumours and sometimes in cells which had morphological characteristics of microglia. Moreover, sometimes strong RCAS1 positivity has been found in metastatic carcinomas. Whether expression of RCAS1 in macrophages accompanying brain tumours is of any importance it is not possible to ascertain at present. However, when elucidating the possible role of RCAS1 in tumour biology, it seems to be necessary to include its presence not only in neoplastic cells.

Key words: *glioma, astrocytoma, RCAS1, brain metastases, immunofluorescence.*

Introduction

Malignant tumours at least at some stage of malignant transformation are able to evade immune surveillance of the host. They possibly do it in different ways but probably the most important mechanisms of their defence against the immune response are the factors either secreted or bound to the tumour membrane that may actively "silence" the immune cells, for instance by induction of their apoptosis. These factors may include protein RCAS1 – a membrane molecule which may induce apoptotic cell death of lymphocytes including T, B and NK cells [12]. RCAS1 (denoting "receptor binding cancer antigen expressed on SiSo cells") for the first time was described by Sonoda as a "novel tumour associated antigen" discovered in cell line SiSo derived from adenocarcinoma of uterine cervix [16,21]. The SiSo cell line had been established by Sonoda and co-workers in 1995 [16]. RCAS1 is recognized by monoclonal antibo-

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dy MoAb22-1, which reacted with SiSo cells. Sonoda and co-workers showed that antigen recognized by antibody MoAb22-1 is present on the surface of ovarian as well as uterine carcinoma cells and that the level of expression is especially marked in invasive cancer [16,17]. Apart from uterine cancers expression of RCAS1 has been reported in many other tumours such as pulmonary, oesophageal, and pancreatic cancers [3,4,11,18,25]. It is possible that RCAS1 when present on the surface of different neoplastic cells may be engaged in the "escape" of the tumour from the immunological surveillance of the host, acting via the receptor on T lymphocytes [19]. Apart from tumours, a possible role of RCAS1 in immunological processes associated with pregnancy has been reported [23,24]. Nakabayashi and co-workers also reported expression of RCAS1 in gliomas, suggesting its role in the mechanism of immune escape by these tumours [10]. There also exists a report of Chinese authors but available in English only in abstract [15]. As already mentioned, strong expression of RCAS1 has been found in many malignant tumours, especially carcinomas, but according to our knowledge little attention, if any, has been paid to metastases. The question arises whether the expression of RCAS1 (if present) can be somehow related to the immunological response of the host. Our preliminary study showed sometimes strong expression of RCAS1 (detected immunohistochemically) in metastatic carcinomas of the brain and also in gliomas. We did not however find evidence that there is a correlation between lymphocytes infiltrate within the tumour and the level of RCAS1 expression [1]. We turned our attention to the strong expression of RCAS1 in non-neoplastic cells, apparently macrophages. The expression was apparently even stronger than in many neoplastic cells. Tumour-infiltrating macrophages seem to be not only secondary bystanders or just scavengers but may play an active role in tumour progression [13]. Here especially interesting is the finding that tumour-associated macrophages may suppress adaptive immunity inducing apoptosis of CD8+ T cells and promote angiogenesis [7,14]. In this context it seems noteworthy to recollect the already mentioned putative role of RCAS1 in evasion of the immune reaction by tumours and also the recent report of Sonoda et al., who have found evidence of a role of RCAS1 in regulation of angiogenesis in tumours [20]. The aim of this study was to find more convincing evidence that RCAS1 co-localizes with macrophages using double immunofluorescence with two antibodies: against RCAS1 and CD68 (a transmembrane glycoprotein, marker of macrophages). Moreover, we wanted to check whether there exists co-localization of RCAS1 with CD74 antigen, which is primarily expressed on antigen-presenting cells, including B cells, monocytes/macrophages and dendritic/microglial cells (and is called "invariant polypeptide of major histocompatibility complex, class II antigen-associated"), and plays a role in chaperoning MHC class II molecules from the endoplasmic reticulum to the cell surface [22].

Material and Methods

Twenty cases of primary (gliomas: 6 grade III and 14 grade IV) and 20 metastatic (all including carcinomas) human brain tumours diagnosed neuropathologically in the Department of Neuropathology were investigated. The protocol of indirect double immunostaining was employed to demonstrate the expression of RCAS1 in cells of microglia and macrophage immunophenotype (CD68 and CD74). Paraffin sections were dewaxed and rehydrated. For antigen unmasking the slides were microwaved in citrate buffer 2 × 5 min and then they were pre-incubated in antibody diluent (DAKO, Glostrup, Denmark, # S0809) for 30 min to reduce non-specific binding and to increase penetration of the antibodies. For simultaneous demonstration of two antigens in the combinations RCAS1/CD68, RCAS1/CD74, and RCAS1/GFAP, a double immunofluorescence procedure using primary antibodies raised in different species (RCAS1 – mouse monoclonal; CD68, CD74, and GFAP – rabbit polyclonal) was applied. The slides were incubated overnight in humid chambers at room temperature with the mixtures of two primary antibodies: (RCAS1, clone 22-1-1, Medical and Biological Laboratories, Naka-ku Nagoya, Japan, # D060-3, diluted 1 : 1000; CD68, Santa Cruz, Santa Cruz, CA, # sc-9139, diluted 1 : 100; CD74, Sigma, St. Louis, MO, # HPA010592, diluted 1:250, GFAP Sigma St. Louis, #G9269 diluted 1 : 100). After rinsing in PBS slides were incubated with secondary antisera: (1) Cy3-conjugated goat anti-mouse IgG (# 115-165-146, diluted 1 : 800), (2) Cy2-conjugated goat anti-rabbit IgG (# 111-225-144, diluted 1 : 200; both from Jackson ImmunoResearch, West Grove, PA). Primary and secondary antibodies were diluted in the same diluent as used for pre-incubation. After a final rinse in PBS the slides were mounted in glycerin/PBS medium.

Microscopy examination. A Nikon *Eclipse* 80i epifluorescence microscope equipped with mercuric burner and filter sets G-2A and B-2 E/C for detection of Cy3 (red) and Cy2 (green) fluorescence respectively was used to examine the immunostaining. Images were recorded using a Nikon DS-Fil digital camera and stored as TIFF files.

Results

In all investigated tumours RCAS1 antibody "stained" large macrophages in which it co-localized with CD68 and CD74. The largest CD68+ macrophages with ample, coarsely granular cytoplasm, apparently representing so-called "gitter cells", were almost uniformly strongly RCAS1 positive both in carcinomas and gliomas (Figs. 1 and 6 respectively). Large CD68+ macrophages were also CD74 positive (Fig. 5). However, this is the conclusion resulting from comparison of corresponding places on slides from the same specimen, since in the double immunofluorescence CD68 and CD74 antibodies were "paired" with RCAS1, not with each other. Only rarely RCAS1 co-localized with CD68 in medium or small cells (Fig. 4) and as

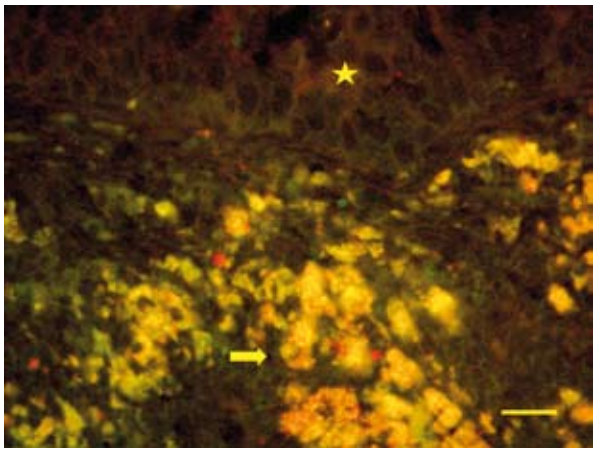

Fig. 1. RCAS1 (red), CD68 (green). CD68 co-localizes with RCAS1 in large macrophages (arrow), so-called "gitter cells", forming clusters in the vicinity of carcinoma cells (asterisk). Bar = 20 µm.

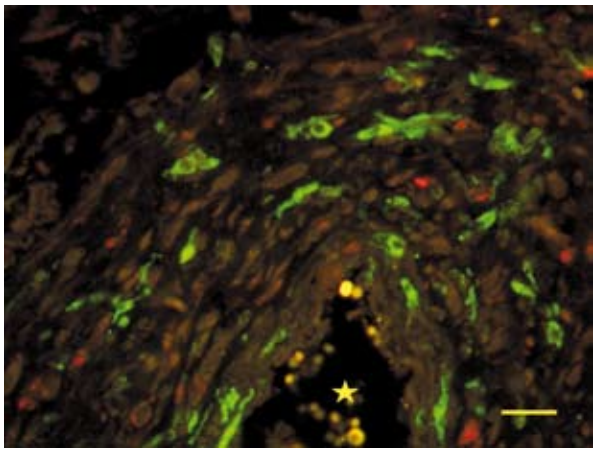

Fig. 2. RCAS1 (red), CD74 (green). CD74 positive, RCAS1 negative reactive microglia around vessel (asterisk). Case of glioblastoma multiforme. Bar = 20 µm.

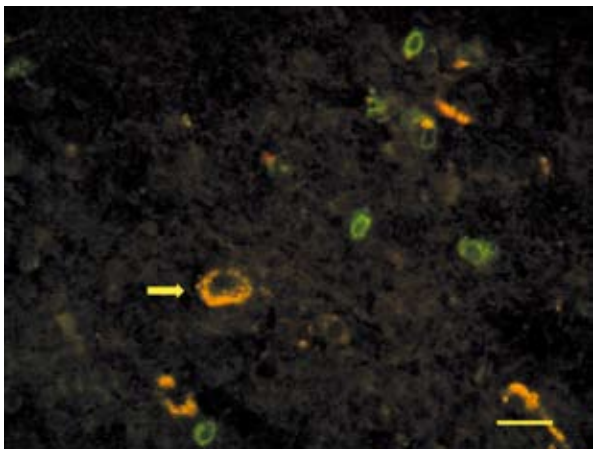

Fig. 3. RCAS1 (red), CD74 (green). Unspecific fluorescence in red channel of lipofuscin in neuron (arrow). A few small CD74+ cells probably represent (quiescent) microglia in the cerebral cortex in the vicinity of glioblastoma infiltration. Bar = 20 µm.

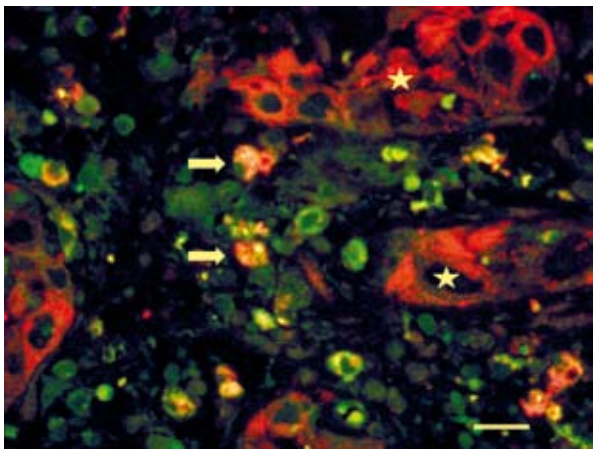

Fig. 4. RCAS1 (red), CD68 (green). RCAS1 co-localizes with CD68 only in some small size CD68+ macrophages (arrows) dispersed among carcinoma cells (asterisks). Bar = 20 µm.

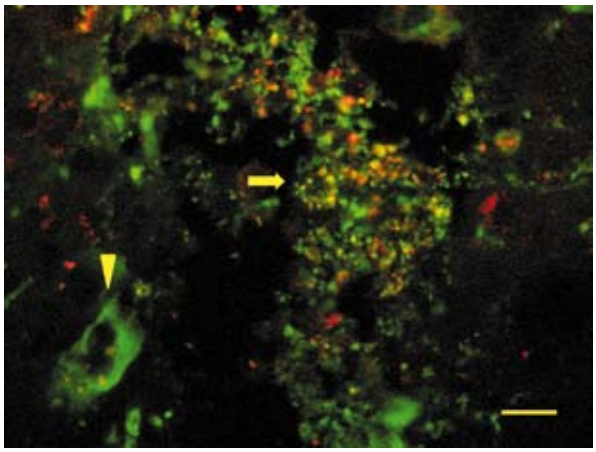

Fig. 5. RCAS1 (red), CD74 (green). Large macrophages in the cluster inside metastasis of carcinoma are CD74 positive (arrow). Large atypical carcinoma cell is also CD74+ but RCAS1- (arrowhead). Bar = 20 µm.

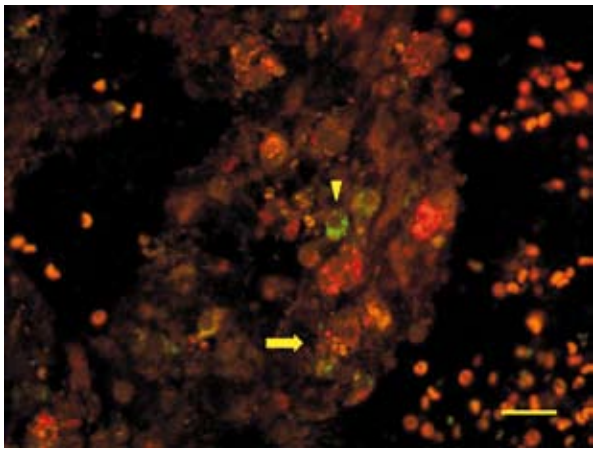

Fig. 6. RCAS1 (red), CD68 (green). RCAS1 co-localizes with CD68 in large macrophages (arrow) but some smaller CD68+ macrophages (arrowhead) either do not express RCAS1 or the expression is remarkably weaker than that of CD68. Case of glioblastoma. Bar = 20 µm.

a result, the large macrophages were practically the only CD68 or CD74 positive cells co-localizing with RCAS1. Other cells positive for RCAS1 were carcinoma (Fig. 7) and glioma cells, the latter co-localizing with GFAP – a marker of astroglia (Fig. 8). In cortical areas not infiltrated by tumour, large neurons showing unspecific fluorescence of lipofuscin could be mistaken for "large macrophages" (Fig. 3). The issue of unspecific autofluorescence is crucial for the proper interpretation of results of immunofluorescence and not only neurons may show this "nasty" feature. At some point we suspected that especially large macrophages ("gitter cells") may also autofluoresce. Therefore we compared the specimens with immunofluorescence with the "control" slides of the same tumours processed identically except for the primary and secondary antibodies. Next, digital photographs of the corresponding places on control and "fully processed" slides were taken, with precisely the same

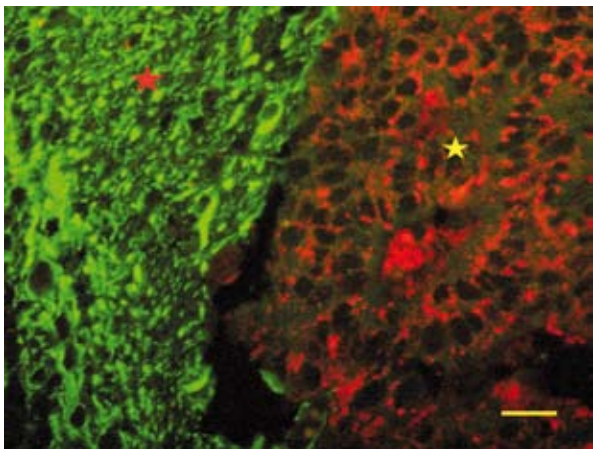

Fig. 7. RCAS1 (red), GFAP (green). Relatively strong expression of RCAS1 in large clusters of carcinoma cells (yellow asterisk) and reactive astrocytic gliosis (red asterisk). Bar = 20 µm.

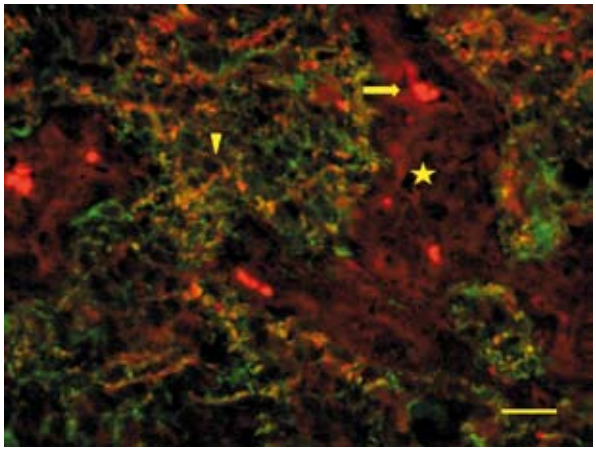

Fig. 8. RCAS1 (red), GFAP (green). RCAS1 in the form of tiny grains and specks (arrowhead) co-localizes with GFAP. A considerable part of the area is occupied by endothelial proliferation (asterisk), negative for GFAP and with only gentle background staining and with unspecific autofluorescence of erythrocytes (arrow). Case of glioblastoma. Bar = 20 µm.

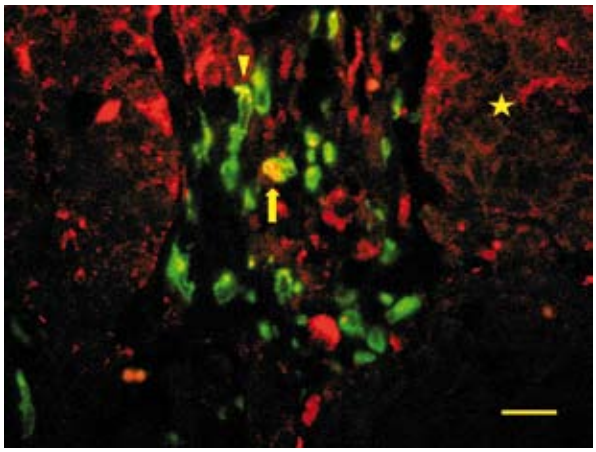

Fig. 9. RCAS1 (red), CD74 (green). Small or medium size CD74+ cells gathering in an area between clusters of carcinoma cells showing some positivity for RCAS1 (asterisks). One of the CD74+ cells apparently is also RCAS1+ (arrow). Noteworthy is an elongated "rod cell" (arrowhead). Bar = 20 µm.

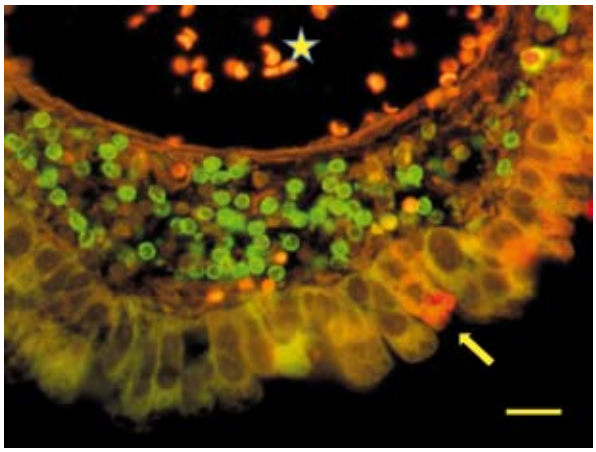

Fig. 10. RCAS1 (red), CD74 (green). Strongly CD74+ small round cells devoid of any processes corresponding to lymphocytes infiltrating tumour (carcinoma). The cells are localized in the space between a markedly dilated blood vessel (asterisk) and a compacted layer of carcinoma cells (one of them notably showing RCAS1 positivity in cytoplasm – arrow). Bar = 20 µm.

camera settings (time of exposure, etc.). Evidently large macrophages which exhibited some autofluorescence unequivocally presented a stronger signal in slides where full immunofluorescence was performed. Macrophages CD68+ were much more numerous in metastatic tumours than in gliomas. CD68- but CD74+ and supposedly microglia-derived cells were typically small or medium size. They were more or less uniformly scattered within gliomas and brain tissue free of neoplasm. In metastatic cancers they usually gathered between clusters of carcinoma cells (Fig. 9). The population of CD74+ cells was morphologically heterogeneous. Apart from the above-mentioned difference in size, there were marked differences in their shape. The smallest, round or oval shape cells with very scarce cytoplasm and apparently devoid of processes (Fig. 10), probably represent lymphocytes, especially B cells, since these cells are

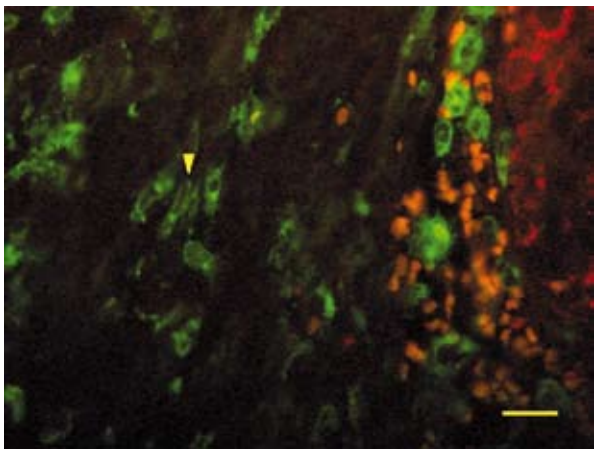

Fig. 11. RCAS1 (red), CD74 (green). Different morphological types of CD74+, some of them with characteristically elongated, rod-like morphology (arrowhead). None of the CD74+ cells is RCAS1+. Case of glioma gr. III. Bar = 20 µm.

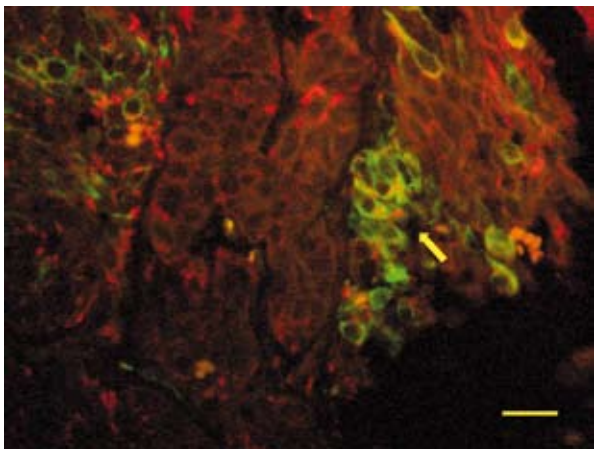

Fig. 12. RCAS1 (red), CD74 (green). CD74 co-localizes with RCAS1 in some carcinoma cells (arrow). Bar = 20 µm.

known to express CD74 [27]. Some have processes but they are very slender. Still others also had more conspicuous cytoplasm and usually thicker processes (Fig. 2). Moreover, there was a separate subpopulation of CD74+ cells that showed characteristically elongated, rod-like morphology (Fig. 11). Different morphological phenotypes of CD74+ cells described above represent almost certainly at least different activation stages of microglia and some also different types of cells (B cells). Numerous carcinoma cells also showed CD74 immunopositivity, sometimes in larger clusters of cells (Fig. 12).

Discussion

Our investigations indicate that at least some macrophages accompanying/infiltrating brain tumours express protein RCAS1. What is the role of activated microglia/macrophages present within and in the vicinity of brain tumour, whether they are merely scavengers or play some more important function in the host-tumour relationship, has not as yet been definitely elucidated. The cross-talk between macrophages and tumour cells may be suppressing but also promoting tumour growth [13]. One cannot exclude either possibility. Stimulated microglial cells are transformed functionally and morphologically from the "quiescent", resting state of cells with delicate, slender processes to the activated phase of ramified, ameboid, and rod cells [6,8]. When activated, they become enlarged with marked cytoplasm and coarser but rather shorter and less numerous processes. The end stage of transformation of microglia represents phagocyting macrophages (sometimes called "gitter cells"). Expression of RCAS1 by tumour-associated macrophages in the brain, to our best knowledge has not been reported so far; however, RCAS1 expression in macrophages elsewhere, especially in bone marrow, was reported [9]. If RCAS1 protein is truly involved in the evasion of immune surveillance by tumours as suggested in several reports, then the presence of RCAS1 in large macrophages could indicate the possibility that macrophages may take part in the mechanism of such evasion. The fact is that the large macrophages show especially strong RCAS1 positivity. The strong signal of expression of RCAS1 in macrophages could be matched only by the one which was observed in some carcinoma cells. As a result, one cannot exclude that macrophages may be involved in putative RCAS1-dependent mechanisms of immune evasion. Numerous apparently microglial cells at different stages of activation (though RCAS1 negative) including elongated "rod cells" showed strong positivity for CD74 (Figs. 2, 11). CD74 blocks the peptide-binding site of MHC class II molecules during its transfer from the endoplasmic reticulum to the cell surface [2]. Probably CD74 also plays a role in signal transduction since it mediates high-affinity binding of the pro-inflammatory cytokine macrophage migration inhibitory factor (MIF) [5]. Regarding the well-known fact that microglia express MHC class II antigens, one may justifiably speculate that CD74 may be of great importance for these cells. However, CD74 expression does not always correlate with that of class II molecules, and also not all activated microglia are MHC class II positive [26,27].

Conclusions

Tumour-associated microglia/macrophages express RCAS1 but it is limited to relatively large and the largest macrophages. Quiescent or reactive microglial cells are apparently RCAS1-negative. Whether expression of RCAS1 in macrophages accompanying brain tumours is of any importance is not possible to ascertain at present. However, when elucidating the possible role of RCAS1 in tumour biology it seems to be necessary to include its expression not only in neoplastic cells.

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