

The immunohistochemical expression of calcitonin receptor-like receptor (CRLR) in human gliomas

L Benes, C Kappus, G P McGregor, H Bertalanffy, H D Mennel, S Hagner

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Correspondence to:
Dr L Benes, Klinik für Neurochirurgie der, Philipps-Universität Marburg, Baldinger Str., D-35043 Marburg, Germany; benes@med.uni-marburg.de

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Background: Gliomas are the most common primary tumours of the central nervous system and exhibit rapid growth that is associated with neovascularisation. Adrenomedullin is an important tumour survival factor in human carcinogenesis. It has growth promoting effects on gliomas, and blockade of its actions has been experimentally shown to reduce the growth of glioma tissues and cell lines. There is some evidence that the calcitonin receptor-like receptor (CRLR) mediates the tumorigenic actions of adrenomedullin.

Aim: To determine whether CRLR is expressed in human gliomas and the probable cellular targets of adrenomedullin.

Methods: Biopsies from 95 human gliomas of varying grade were processed for immunohistochemical analysis using a previously developed and characterised antibody to CRLR.

Results: All tumour specimens were positive for CRLR. As previously found in normal peripheral tissues, CRLR immunostaining was particularly intense in the endothelial cells. This was evident in all the various vascular conformations that were observed, and which are typical of gliomas. In addition, clear immunostaining of tumour cells with astrocyte morphology was observed. These were preferentially localised around vessels.

Conclusions: This study has shown for the first time that the CRLR protein is present in human glioma tissue. The expression of the receptor in endothelial cells and in astrocytic tumour cells is consistent with the evidence that its endogenous ligand, adrenomedullin, may influence glioma growth by means of both direct mitogenic and indirect angiogenic effects. CRLR may be a valuable target for effective therapeutic intervention in these malignant tumours.

Malignant gliomas are the most common primary tumours of the central nervous system with a median mortality of between nine and 14 months.¹ They are characterised by rapid growth, invasion into the surrounding normal brain tissue, and by neovascularisation, which is inversely related to prognosis.² The different grades (World Health Organisation (WHO) II–IV) of glioma are thought to reflect progressive anaplasia, with an increasing degree of normal and pathological vessel formation.³ Targets for therapeutic intervention need to be identified.

Adrenomedullin and calcitonin gene related peptide (CGRP) are related members of the calcitonin family of regulatory peptides, which also includes amylin and calcitonin.⁴ Both peptides are widely distributed in various peripheral tissues and the central nervous system, and induce a wide variety of biological effects.⁵ Adrenomedullin has also been identified as playing an important role in tumour pathogenesis.^{6–8} The calcitonin receptor-like receptor (CRLR), a G protein coupled receptor, acts as a receptor for both peptides, which explains their partly shared biological actions.^{9–10} However, the interaction of adrenomedullin and CGRP with this receptor depends upon the coexpression of a so called receptor activity modifying protein (RAMP).¹¹ Whereas coexpression of CRLR with RAMP-2 or RAMP-3 generates an adrenomedullin receptor, coexpression with RAMP-1 generates a CGRP receptor.

Having developed an antibody to human CRLR,¹² we obtained the first definitive evidence of a broad distribution of this receptor protein in peripheral tissues,^{13–14} in particular the microvascular endothelium. Because the synthesis and release of both peptides appears to occur in response to noxious and inflammatory stimuli, this peptide–receptor system may mediate important changes in the microvasculature under certain pathophysiological conditions. In particular, it is of interest to study the role of this system in the vascular responses to tissue ischaemia and hypoxia, which are conditions that cause upregulation of adrenomedullin.¹⁵ Hypoxia induced vascular changes, including neovascularisation, have been well characterised in human adult supratentorial gliomas. Therefore, the aim of our study was to establish whether the CRLR protein is widely expressed in human gliomas and to determine its cellular distribution in these tissues.

MATERIAL AND METHODS

Tumour biopsies were obtained from routine neurosurgical management. Tumours were usually preserved in their native state and processed as frozen sections, fixed in buffered formalin and glutaraldehyde for neuropathological diagnoses using routine histological methods.

“Hypoxia induced vascular changes, including neovascularisation, have been well characterised in human adult supratentorial gliomas”

Abbreviations: CGRP, calcitonin gene related peptide; CRLR, calcitonin receptor-like receptor; GFAP, glial fibrillary acid protein; RAMP, receptor activity modifying protein; WHO, World Health Organisation

Table 1 Distribution of main diagnoses in the sample of investigated tumours according to the latest World Health Organisation classification

Diagnosis	Number (N = 95)
Pleomorphic xanthoastrocytoma	3
Pilocytic astrocytoma	18
Fibrillary astrocytoma	14
Gemistocytic astrocytoma	3
Anaplastic astrocytoma	18
Glioblastoma	39

Using immunohistochemistry, 95 single specimens were analysed for CRLR immunostaining and directly compared with the staining pattern of glial fibrillary acid protein (GFAP). Table 1 gives the histological WHO classification of the selected tumours.

Development, purification, and application of antibodies

The raising of rabbit antisera against the commercially synthesised human CRLR sequence conjugated with thyroglobulin and mixed with Freund's adjuvant has been described in detail previously.¹² One antiserum MR 567 gave best results in western blotting and immunohistochemistry and was therefore affinity purified.

Immunohistochemistry

Immunohistochemistry was performed on paraffin wax embedded 1–2 µm sections, mounted on SuperFrost®Plus slides. After dewaxing and rehydration, sections were microwaved for 20 minutes to assist antigen exposure. After rapid cooling and two washes, the sections were incubated with primary antibody for 30 minutes at room temperature. The most effective dilution of the antibody in this series of staining procedures was 1/100 to 1/200. The routinely used peroxidase–antiperoxidase method,¹⁶ which involves the use of three subsequent antibodies and diaminobenzidine as chromogen, was used for visualisation of the antigens.

Evaluation

The various patterns of CRLR immunostaining were evaluated, including the relative number of CRLR immunostained tumour cells and the degree of co-staining with the astrocyte marker, GFAP. The counting device was a simple quadrangular intersectional microscopic ocular counting field. The clinical classification of the tumour tissue was determined by one of the authors (HDM), according to the rubrics and grading scheme of the most recent issue of the WHO brain tumour classification.¹⁷

RESULTS

In human gliomas, CRLR immunostaining was seen predominantly in two cell types; namely, vascular endothelial cells and astrocytic tumour cells. In addition, neuronal perikarya were also immunostained in well preserved neighbouring cortical tissue.

Vessels

Gliomas of increasing malignancy produce a wealth of blood vessels of normal and pathological morphology. Various vessel types exhibiting CRLR immunoreactivity could be distinguished. In low grade astrocytomas, larger veins stain positively for CRLR (fig 1A) and, therefore, could be distinguished from the small vacuoles that are usually formed by this tumour type. In highly aggressive gliomas

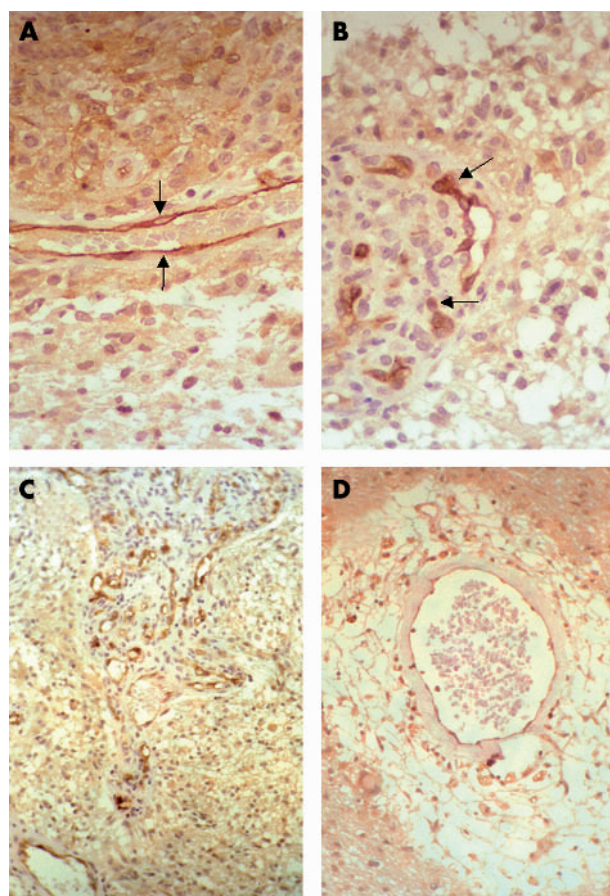


Figure 1 (A) Large vein (arrows) within low grade part of a glioma. Note the positive astrocytic extensions in the upper half of the micrograph. The endothelial lining shows equally strong immunostaining; original magnification, $\times 500$. (B) Small vessel conglomerate within a glioblastoma. The arrows point to cells expressing calcitonin receptor-like receptor (CRLR) protein, which are remote from the direct endothelial lining. Nearby astrocytes again show positive staining; original magnification, $\times 500$. (C) Formation of capillary garlands with numerous interweaving capillary lumina depicted by the positive CRLR immunostaining of endothelia. Unstained cells obviously belonging to the vessel wall proliferation (see text) are seen in close proximity; original magnification, $\times 250$. (D) Large vessel with concomitant smaller and branching secondary vessels. Note the unstained fibrous vessel wall and some cells gathering around the larger vessel that equally express the receptor protein; original magnification, $\times 500$

(WHO III and IV), blood vessel proliferation may start with endothelial growth, which could be detected by the presence of a double layer of endothelial cells undergoing mitosis (fig 1B). Garland-like capillary and angiomatous vessel formations, typical of malignant gliomas, were clearly CRLR immunostained, whereas adjoining tumour cells were unstained or only slightly stained (fig 1C, D). Although vessels were surrounded by CRLR immunostained tumour cells, the vascular contours could be clearly distinguished from these tumour cells, because the CRLR immunostaining was evidently restricted to the endothelial layer of the vessels missing in the adjacent vessel walls.

Tumour cells

Tumour cells were stained inconsistently, but clear and distinctive CRLR immunostaining of single tumour cells, in addition to small and large clusters of tumour cells, could be seen frequently. Different patterns of CRLR immunoreactivity in tumour cells could be identified. Single immunostained

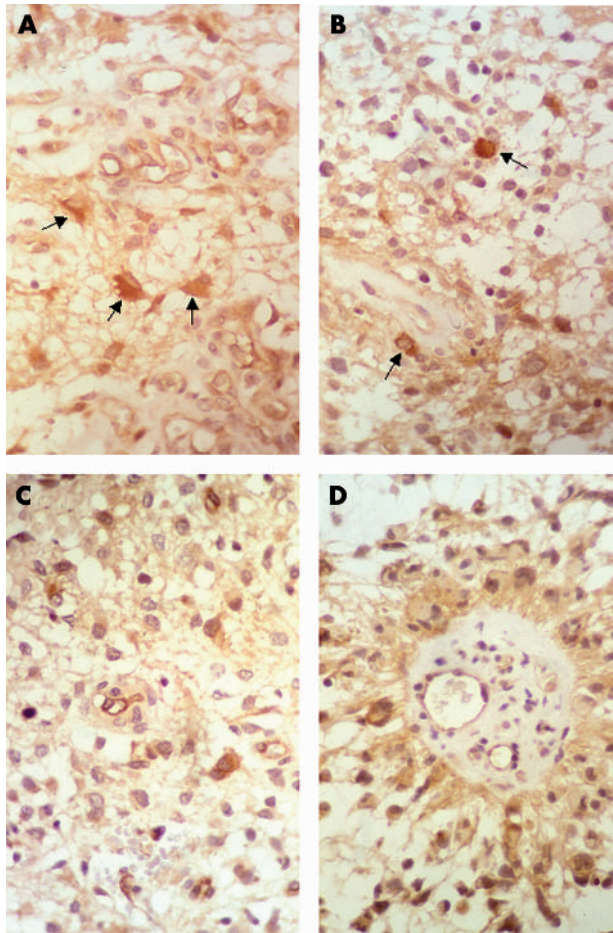


Figure 2 (A) Three calcitonin receptor-like receptor (CRLR) immunostained cells (arrows) with a typical astrocytic shape between two fields of vascular proliferation (upper and lower third); original magnification, $\times 850$. (B) Astrocytic cells around the vessel wall (lower left), directly adjacent or at some distance (arrows). Few scattered positively stained cells in the remaining tumour; original magnification, $\times 500$. (C) Scattered positively stained cells within the loose network of a fibrillary astrocytoma. The endothelia of a few vessels and tumour cells are stained positively for CRLR; original magnification, $\times 500$. (D) Tumour cells around a pathological vessel in a glioblastoma. The “reduplicated” lumina are lined by CRLR positive endothelial cells. Note all perivascular astrocytes are stained; original magnification, $\times 850$.

cells, within a rather inconspicuous surrounding, exhibited cytoplasmic extensions and were recognisable as typically shaped astrocytes (fig 2A). They correspond to the protoplasmic subtype common in reactive and tumorous conditions. These were found to be preferentially aggregated around vessels and a small distance from the vessel (fig 2B).

Some, but not all, “stuffed” gemistocytes were intensely CRLR immunoreactive. These cells occur in small clusters, similar to other multipolar tumour cells, and possibly represent focal cellular multiplication.

A further pattern of expression consisted of diffuse staining in most of the newly growing tumour cells. This was recognisable in the form of a very “loose network” of low grade fibrillary astrocytic cells (fig 2C), in addition to a much denser bipolar arrangement of anaplastic cells, and in the variegated cellular morphology pattern of highly malignant glial tumour cells around pathological vessels (fig 2D).

Neurones

Cortical neurones, especially the perikarya, presumably entrapped in the tumour itself, also exhibited strong and

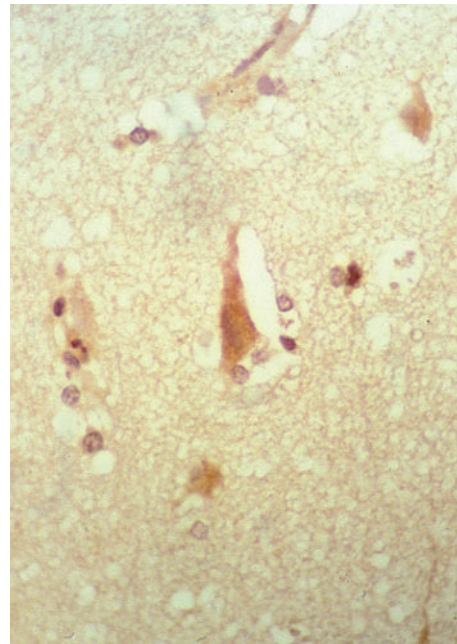


Figure 3 Neuronal perikarya, especially within the deep cortical layers, are calcitonin receptor-like receptor immunoreactive; original magnification, $\times 500$.

constant CRLR immunoreactivity (fig 3). Most of them had an altered shape, presumably as a result of the adjacent tumour tissue acting upon them.

Cellular localisation and correlation of relevant structures

Staining in all cases was within the confines of the perinuclear cytoplasm. This held true for neuronal perikarya, endothelial cells, and astrocytes. However, astrocytes did not show a constant intensity of immunostaining. Some giant tumour cells were stained in the periphery although the protoplasmic centre was negative. Gemistocytes displayed glial fibrillary acid protein and CRLR immunoreactivity. Using pertinent aniline dyes and GFAP immunostaining, the restriction of CRLR antigen to a subpopulation of astrocytes and endothelial lining could be demonstrated.

Morphometric and statistical results

Numbers of GFAP positive cells in defined areas of gliomas of different grades were counted and compared with CRLR positive cell numbers in the randomly selected fields. There was a small decrease in the proportion of GFAP immunostained cells with ascending grade of malignancy (glioma II (mean, 24.8%; SD, 14.8%), glioma III (mean, 19.8%; SD, 10.2%), and glioblastoma (mean, 14.8%; SD, 9.5%)). No correlation was found for CRLR immunostained cells. The highest numbers of CRLR immunostained cells were found in glioma III tumours (mean, 41.8%; SD, 33.6%).

DISCUSSION

CRLR is the cognate receptor of two vasoactive peptides—adrenomedullin, which is produced by a variety of tissues and cells in the cardiovascular system, and CGRP, which is produced by perivascular nerves.^{18 19} We have recently used our anti-CRLR antibody to demonstrate the widespread distribution of CRLR in peripheral tissues¹⁴ and its prevalence in the microvasculature. Here, we have demonstrated the distribution of CRLR in human gliomas of different grades, and have shown that it is expressed by different cell types

within the tumour tissue. To our knowledge, these are new findings, and they suggest that CRLR plays an important role in the pathology of human gliomas. In particular, it is important to view our findings in light of the well documented evidence that adrenomedullin may play an important role in glioma tumorigenesis. The presence of CRLR immunoreactivity in the vascular endothelium and in astrocytic tumour cells is consistent with the notion that adrenomedullin may influence glioma growth by means of both direct mitogenic and indirect angiogenic effects.²⁰

It has been proposed by Folkman that tumour growth depends on new vessel growth,²¹ and recent studies²² indicate that the growth of gliomas exhibits two growth phases. Initially, normal cerebral vessels are recruited to support early tumour growth. The second phase is characterised by angiogenesis and neovascularisation, which appears to be preceded by transient apoptosis of the pre-existing vascular cells. Both adrenomedullin²³ and CGRP²⁴ have been shown to promote endothelial growth in in vitro studies of human umbilical vein cells. In addition, there is in vivo evidence of adrenomedullin acting as a mediator of physiological angiogenesis.²⁵ Furthermore, vasodilation is a key early process in angiogenesis.^{26–27} Both adrenomedullin and CGRP are potent vasodilators. The high amounts of CRLR protein that we found in the endothelial cells of the various vascular conformations of human glioma suggest that CRLR plays a role in angiogenesis in gliomas. This should be further evaluated, in addition to the respective roles of adrenomedullin and/or CGRP.

“Our immunohistochemical analysis did not indicate a clear correlation between the concentration of the calcitonin receptor-like receptor and the tumour grade”

Recently, it has become increasingly evident that adrenomedullin plays an important role in tumour pathogenesis.^{7–8, 28–30} The expression of this endogenous CRLR ligand has been demonstrated in normal and neoplastic cells, including gliomas. Adrenomedullin acts as a glioma growth factor^{20–31} via direct (possibly autocrine) actions, and by promoting vascularisation of glioma tissue. Our findings concerning the presence of CRLR protein in glioma tumour cells and vasculature are consistent with these modes of action.

Real time quantitative polymerase chain reaction has shown that the degree of expression of adrenomedullin in human gliomas correlates with the grade of tumour, with especially high expression in grade IV.²⁰ Our immunohistochemical analysis did not indicate a clear correlation between the concentration of the CRLR protein and the tumour grade. This suggests that the degree of vascularisation and tumour differentiation may not depend on concentrations of CRLR, but on those of its ligand adrenomedullin. In addition, the

expression of RAMPs and their degree of expression in gliomas in relation to the tumour grade need to be considered in further studies.

Regions of hypoxia are common in solid tumours and are associated with the transcription of a battery of genes, including adrenomedullin in tumour cells.¹⁵ Adrenomedullin is thought to be a mediator of hypoxia driven angiogenesis,³² which is a major factor in tumour growth. It has been shown to protect tumour cells from hypoxia induced apoptosis, probably via activation of CRLR.³³ The antiapoptotic effects of adrenomedullin explain (at least partly) the mitogenic actions of adrenomedullin, which have been demonstrated in a variety of non-tumour and tumour cells.⁸

Our morphometric and statistical results comparing GFAP and CRLR containing neoplastic astrocytes lead to the notion that neoplastic glial cells expressing CRLR form a subset of astrocytic tumour cells mainly expressed in glioma grade III neoplastic astrocytic tumour cells.

Apparently normal cortical perikarya within the glioma tissues were also found to express CRLR protein, which is consistent with earlier findings of the presence of CGRP and CGRP binding sites within the human cerebral cortex.^{34–35} Adrenomedullin has also been detected in rat cerebral cortical neurones (in pyramidal cells and small cells).³⁶

In conclusion, in our study we have demonstrated the wide distribution of CRLR in different grades of human glioma, especially in vessels, astrocytes, and neurones. Our findings are particularly important for glioma biology because CRLR may play a major role at distinct stages of angiogenesis and carcinogenic tumour progression, and may be a useful basis for further studies on this topic.

Authors' affiliations

L Benes, C Kappus, H Bertalanffy, Department of Neurosurgery, Philipps-University Marburg, D-35043 Marburg, Germany
H D Mennel, Department of Neuropathology, Philipps-University Marburg
G P McGregor, S Hagner, Institute of Physiology, Philipps-University Marburg

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Take home messages

- We have shown for the first time that the calcitonin receptor-like receptor (CRLR) is present in human glioma tissue
- The receptor is particularly expressed in endothelial cells and astrocytic tumour cells, consistent with the notion that its endogenous ligand, adrenomedullin, may influence glioma growth by means of both direct mitogenic and indirect angiogenic effects
- CRLR could possibly be a valuable target for effective therapeutic intervention in glioma

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