UNIVERSITE DE LA MEDITERRANEE-AIX-MARSEILLE II

FACULTE DE MEDECINE

ECOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTE

THESE DE DOCTORAT
Spécialité : NEUROSCIENCES

Présentée publiquement par

Philippe METELLUS

Pour obtenir le grade de

Docteur de l'Université de la Méditerranée

(Arrêté du 30 Mars 1992)

Rôle de l'adrénomédulline dans la néoangiogenèse et l'invasion tumorale

Présentée et soutenue le 19 Décembre 2011 devant le jury composé de :

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Pr. Loiseau Hugues	Rapporteur
Pr. Figarella-Branger Dominique	Président du Jury
Pr. Ouafik L'Houcine	Directeur de thèse

UMR911-Equipe 4
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Je dédicace ce travail à mes parents, à mon épouse, Isabelle Métellus, et à mes 3 enfants, Paul, Camille et Marion

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Sommaire

Liste des Abréviations

AM :adrénomédulline
AMPc :Adénosine monophosphate cyclase
AMR : recepteur de l'adrénomédulline
bFGF ;basic fibroblast growth factor
CE: Cellules endothéliales
CGRP:calcitonin gene related peptide
CDK: cyclin dependant kinase
Cox-2: cyclo-oxygenase -2
CRL:calcitonin receptor like
EGF:endothelial growth factor
EGFR: endothelial growth factor receptor
GPI: glycosylphosphatidylinositol
HIF1α: hypoxia inducible factor 1
HMEC: human microvascular endothelial cell
HRE: hypoxia responsive element
HUVEC: human umbilical vascular endothelial cell
INR: initiator element
IP3K: Inositol trisphosphate kinase
IRM image par resonance magnetique
IDH1: isocitrate deshydrogenase 1
AMBP-1 : adrenomedullin bondong protein 1
IL-1 : interleukine 1
LCR :liquide céphalo-rachidien
MAP kinase : mitogen activated proteins kinases
MEC: matrice extra cellulaire
MMP: matrice metallo protease
MGMT : O6-methylguanine transferase
P : péricyste
PAM :peptidyl glycine α-amidating monooxygenase
PAMP:pro-adrenomedullin 20 peptide
PCR:polymerase chain reaction
PDGF:platelet-derived growth factor
PDGFR:platelet-derived growth factor receptor
PKB: protein kinase B
PGE2 : prostaglandine E2
PTEN: phosphatise and tensine homolog
RAMP: receptor activity modifying protein
RCPG: recepteur couple a la proteine G
RES : responsive element
TGFb : transforming growth factor b
TNF: tumor necrosis factor
VEGF:vascular endothelial growth factor

AVANT-PROPOS

Le cancer touche 12% de la population mondiale et peut dépasser le seuil des 25% dans les pays industrialisés (Stewart et Kleihues, 2005). Il représente désormais la première cause de mortalité en France, devant les maladies cardiovasculaires.

Les tumeurs auxquelles sont associées les pronostics et les survies à cinq ans les plus médiocres ne sont pas forcément celles dont l'incidence est la plus élevée. Le cancer du pancréas et les tumeurs cérébrales de haut grade (notamment les glioblastomes) ont les taux de survie à 5 ans les plus bas. Le cas des tumeurs cérébrales est particulièrement délicat du fait de leur emplacement même. En effet, même les cas classés comme bénins peuvent être situés dans des zones impliquées dans des fonctions essentielles à une vie normale pour le patient rendant l'exérèse chirurgicale difficile voire impossible. Parmi les tumeurs cérébrales, les gliomes malins représentent 45% des tumeurs cérébrales et 85% d'entre eux sont des glioblastomes, les gliomes de plus haut grade de malignité. Malgré l'avancée technologique des méthodes de dépistage et des traitements, le pronostic de ces tumeurs demeure toujours aussi sombre depuis vingt ans.

Le cancer trouve son origine dans une croissance cellulaire incontrôlée et inappropriée dans un tissu ou un organe. Le développement tumoral s'effectue en trois étapes. Tout d'abord, dans la phase d' « initiation », l'accumulation d'altérations génétiques de séquences d'ADN codant pour un protooncogène ou un gène suppresseur de tumeurs entraine la transformation de cellules saines en cellules néoplasiques. Ces altérations étant souvent déclenchées par différents facteurs physiologiques (stress, inflammation chronique,…) et/ou environnementaux (substances cancérigènes contenues dans l'alimentation, pollution, tabagisme,…). Vient ensuite la phase de « promotion » tumorale pendant laquelle la masse tumorale augmente suite à la survie et à la prolifération incontrôlée des cellules néoplasiques. L'environnement tumoral

contribuant également à la progression des tumeurs. De même, le développement, par le phénomène d'angiogenése tumorale, de nouveaux vaisseaux au sein des tumeurs est indispensable à la survie et la croissance de ces dernières. Enfin dans la dernière étape de la « progression » tumorale peut émerger un phénotype malin.

L'inhibition de la formation des vaisseaux tumoraux supprime la croissance tumorale dans des modèles expérimentaux. Cette découverte permet d'espérer qu'en attaquant la vascularisation tumorale, une approche utile dans le traitement du cancer humain pourra être développée : la thérapie anti-angiogénique. Parmi les molécules intervenant dans la néoangiogenèse, des peptides comme le VEGF (vascular endothelial growth factor), le bFGF (basic fibroblast growth factor) sont bien connus. Plus récemment découvert, l'adrénomédulline (AM) est un peptide dont l'expression est corrélée à l'agressivité de certaines tumeurs et qui représente un maillon clé dans les interactions entre les cellules tumorales et les cellules du microenvironnement.

Notre sujet porte sur le rôle de l'AM dans la néoangiogenèse tumorale via les récepteurs CLR/RAMP2 et CLR/RAMP3. Nos travaux établissent un réel constat de l'importance de l'AM dans la stabilisation vasculaire tumorale. Nous verrons que l'AM, ubiquitaire et ″reine″ du microenvironnement tumoral, s'invite à chaque étape essentielle de la néoangiogenèse et de l'invasion tumorale.

INTRODUCTION BIBLIOGRAPHIQUE

PARTIE 1 : Les Glioblastomes

I. Généralités sur les gliomes

Les cellules gliales

Deux grands types cellulaires cohabitent au sein du système nerveux central (SNC) : les neurones, qui représentent 10% du capital cellulaire du SNC, et les cellules gliales, qui représentent 90% du SNC.

Les cellules gliales sont regroupées sous le terme de glie et jouent un rôle de nutrition, de protection et de soutien auprès des neurones et sont classées en quatre types histologiques : les cellules épendymaires, les astrocytes, les oligodendrocytes et les cellules microgliales. Chacun des différents types cellulaires est susceptible de subir des dysfonctionnements pouvant engendrer le développement d'une tumeur.

Les gliomes

Si au sens général les gliomes incluent toutes les tumeurs dérivées des cellules gliales, au sens plus restreint, il s'agit de tumeurs primitives du SNC dérivées des astrocytes et des oligodendrocytes.

Les gliomes représentent les tumeurs primitives les plus fréquentes et les plus graves du SNC. Ils représentent plus des 9/10ème des tumeurs malignes primitives cérébrales. Leur incidence en France est de 2500 à 3000 cas par an.

Caractéristiques générales des gliomes

En raison du volume non extensible de la boîte crânienne, les gliomes peuvent comprimer des zones du cerveau vitales et causer des troubles importants. Le pronostic de ces tumeurs reste sombre. Chaque type tumoral a une localisation topographique et une structure histologique qui lui est propre. On oppose les gliomes infiltrants (envahissant le parenchyme adjacent sain), qui peuvent avoir une structure mixte (solide

et infiltrant) ou être composés uniquement de cellules tumorales isolées et qui peuvent évoluer lentement ou être d'emblée de haut grade, aux gliomes circonscrits, qui ne sont constitués que de tissu tumoral, généralement bénins et représentés par les astrocytomes pilocytiques.

Les gliomes infiltrants s'observent à tout âge, bien que plus fréquents chez l'adulte. La forme la plus fréquente et la plus maligne est le glioblastome (GBM) dont la survie moyenne après exérèse chirurgicale est d'environ douze mois.

II. Les Glioblastomes : origine et classification

1. Généralités sur les glioblastomes

Le GBM est la tumeur primitive du cerveau la plus fréquente, elle est hautement agressive et son pronostic est mauvais. Cette tumeur représente la forme la plus maligne des astrocytomes (astrocytome de grade IV). Elle représente 52% des tumeurs primitives et 20% de toutes les tumeurs intra-crâniennes. La survie médiane est de 6 à 9 mois avec traitement palliatif, 12 mois avec radiothérapie et de 16 mois avec radio et chimiothérapie. La survie post-opératoire varie de 12 mois (50%), 24 mois (20%) à 36 mois (2%). Presque tous les cas de GBM sont sporadiques, sans prédisposition familiale.

2. Classification des gliomes selon l'OMS 2007

La première classification des tumeurs cérébrales par l'OMS est établie en 1979 et a été révisée trois fois (1993, 2000, 2007). Cette classification propose un typage histologique en fonction du type cytologique prédominant (astrocytaire, oligodendrocytaire ou mixte) ainsi que du grade qui lui est déterminé en fonction de la présence ou l'absence de critères histologiques de malignité suivant : densité cellulaire, atypies nucléaires, mitoses, prolifération microvasculaire et nécrose (Louis et al., 2007).

De cette manière, trois groupes de gliomes sont déterminés en fonction de l'origine cellulaire de la tumeur (Tableau 1) : les tumeurs astrocytaires, les tumeurs oligodendrogliales et les tumeurs mixtes oligoastrocytaires. Quatre grades de malignité sont également déterminés. Les gliomes circonscrits donc bénins sont de grade I tandis que les gliomes diffus sont de bas grade (II) ou bien malins de haut grade (III et IV), sachant que les gliomes de bas grade évoluent vers la malignité.

De part leur différence morphologique et leur différence de profil génétique, trois types de GBM sont reconnus dans la classification officielle de l'OMS. Il s'agit du :

- Glioblastome multiforme conventionnel
- Glioblastome à cellules géantes (5% des GBM)
- Gliosarcome (2% des GBM)

Cependant trois autres variantes existent et sont reconnues mais ne sont pas mentionnées dans la classification par manque de recul et d'accord entre les membres participant à la mise en place de cette classification. Il s'agit du :

- Glioblastome à composante oligodendrogliale (HE et al., 2001).
- Glioblastome aux caractéristiques neuronales primitives comme les tumeurs neuroectodermiques (Perry et al., 2009).
- Glioblastome à petites cellules (Miller et al., 2007).

Cette classification présente cependant des insuffisances et son manque de reproductibilité a souvent été souligné (Coons et al., 1997 ; Mittler et al., 1996 ; Figgarella-Branger et Bouvier, 2005). Ainsi l'histologie seule ou associée aux données cliniques et à l'imagerie ne permet pas encore une prise en charge thérapeutique optimale des gliomes.

Les tumeurs astrocytaires	
* Astrocytome cellules géantes sous-épendymaire	Grade I
* Astrocytome pilocytique	Grade I
o Astrocytome pilocytique, variant pilomyxoïde	Grade II
* Astrocytome diffus	Grade II
o Astrocytome fibrillaire	
o Astrocytome protoplasmique	
o Astrocytome gemistocytique	
* Astrocytome anaplasique	Grade III
* Xanthoastrocytome pléiomorphe	Grade II/III
* Glioblastome	Grade IV
o Glioblastome à cellules géantes	Grade IV
o Gliosarcome	Grade III
* Gliomatose cérébrale	
Les tumeurs oligodendrogliales	
* Oligodendrogliome	Grade II
* Oligodendrogliome anaplasique	Grade III
Les tumeurs mixtes oligo-astrocytaires	
* Oligoastrocytome	Grade II
* Oligoastrocytome anaplasique	Grade III

Tableau 1 : Classification des gliomes selon l'OMS 2007

D'après Louis et al., 2007.

3. Principales altérations génétiques et épigénétiques observées dans les glioblastomes

Les principales altérations génétiques observées dans les GBM ont pour cible le cycle cellulaire ou les voies de transmission du signal. Elles consistent essentiellement par le gain ou la perte, totale ou partielle, d'un chromosome, par l'amplification ou la perte d'un allèle spécifique, par une activation ou une inactivation causée par une mutation et enfin par la méthylation d'un promoteur (Kanu et al., 2009).

Les altérations les plus fréquentes au sein des glioblastomes sont :

- La perte de l'hétérozygotie (LOH : Loss Of Heterozygosity) du chromosome 10 (Rasheed et al., 1995).
- La perte du bras chromosomique 9p altérant l'expression des gènes $p14^{ARF}$ et $p16^{INK4a}$
- L'amplification du gène PDGFRA (Platelet Derived Growth Factor Receptor) (Ohgaki et al., 2004).
- Une amplification du gène de l'EGFR (Epidermal Growth Factor Receptor) sur le chromosome 7.
- Des mutations inactivatrices de PTEN (Phosphatase and tensin homolog) au niveau du chromosome 10.

Les GBM secondaires présentent plus fréquemment :

- Des mutations inactivatrices du gène P53 au niveau du chromosome 17.
- Des mutations inactivatrices du gène RB1 (Retinoblastome) au niveau du chromosome 13.
- La méthylation du promoteur sur le chromosome 10q26, du gène de l'enzyme de réparation de l'ADN MGMT (O_6-methyl guanine-DNA methyltransferase).

- Des mutations de IDH1/IDH2 (isocitrate dehydrogénase 1 et 2) (Balss et al., 2008 ; Parsons et al., 2008).

Les mutations de ces gènes sont responsables des caractéristiques propres aux GBM.

III. Caractéristiques de la composante gliale tumorale responsable du phénotype malin des GBM

Nous considérons la composante gliale tumorale des GBM comme l'ensemble des cellules tumorales constituant la tumeur sans les cellules qui constituent les capillaires tumoraux. Pendant le développement tumoral, les cellules acquièrent de multiples mutations génétiques en particulier pendant la première étape d' « initiation » durant laquelle elles acquièrent leur potentiel néoplasique. La seconde étape de « promotion » commence par l'expansion de la population néoplasique initiale et dans la dernière étape de « progression tumorale » émerge le phénotype malin de la tumeur caractérisé dans les GBM par les propriétés suivantes.

1. Prolifération accrue

Contrairement aux neurones, les cellules gliales se divisent. La prolifération anormale des cellules gliales tumorales des GBM est due au dérèglement de l'activité et/ou de l'expression de certaines molécules ayant un rôle dans la prolifération cellulaire (Kanu et al., 2009).

En effet, l'amplification du gène des récepteurs du facteur de croissance EGF, l'EGFR est souvent associée à des altérations structurelles menant à un récepteur constitutivement autophosphorylé donc activé (Ekstrand et al., 1991). De plus, l'expression du récepteur du PDGF, le PDGFR est aberrante (Hermanson et al., 1992). L'activation des gènes de ces deux récepteurs à tyrosine kinase provoque la conversion des Ras-GDP en Ras-GTP (Guha et al., 1997) qui active la voie des MAPK kinases,

provoquant ainsi l'activation de la prolifération des cellules. L'activation de ces récepteurs entraine également l'activation de la PI3K (phosphatidyl-inositol-3-kinase) qui elle-même active la voie de l'Akt. Celle-ci aboutit à la transcription de gènes impliqués dans la promotion de la prolifération des cellules.

De même, le gène suppresseur de tumeur PTEN code pour une protéine à l'activité de tyrosine phosphatase qui inhibe l'activité PI3K en déphosphorylant PIP3 (phosphatidylinositol-3,4,5 triphosphate) et PIP2 (phosphatidylinositol-3,4,5 diphosphate) (Maehama et al., 1998). Or PI3K phosphorylé active l'Akt et module ainsi l'activité des protéines jouant un rôle important dans la prolifération, en activant la transition de la phase G1 à S du cycle cellulaire. En temps normal, PTEN produit donc un blocage du cycle des cellules en G1. De ce fait, la mutation inactivatrice de PTEN aboutit à l'effet inverse avec une prolifération non contrôlée des cellules.

Le TGFβI et son récepteur TGFβRII sont exprimés dans les GBM alors qu'ils ne sont pas dans le cerveau ni dans les gliomes de bas grade (Yamada et al., 1995). Aussi, en réponse à sa stimulation par le TGFβ, le récepteur TGFβRI active indirectement des gènes qui contribuent à la prolifération cellulaire, dont le PDGF-A (Zhang et al., 1997) qui est un facteur prolifératif.

De plus, la protéine Rb issu du gène RB1, inhibe normalement l'action du facteur de transcription E2F1 après interaction avec ce dernier. En phosphorylant Rb, le complexe CDK4/cyclin D1 libère E2F1, qui active alors des gènes impliqués dans la transition de la phase G1 à la phase S du cycle cellulaire (Sherr et al., 1999). L'une des protéines inhibitrices des CDK, la $P16^{INK4a}$, régule cette transition en inhibant le complexe CDK4/Cyclin D1 après liaison au CDK4. L'inactivation du gène de RB1 et la délétion de celui de $P16^{INK4a}$ mènent donc à un cycle cellulaire incontrôlé des cellules tumorales de glioblastomes.

Enfin le facteur P53 est impliqué dans la régulation du cycle cellulaire pendant la phase de réparation et de prolifération. P53 est un facteur de transcription à vie courte, surexprimé après un stress cellulaire. Il facilite la réparation de l'ADN en interrompant le cycle cellulaire pendant l'action des enzymes de réparation, en activant et induisant la transcription d'un inhibiteur des CDK, le facteur P21 qui protège l'action des Rb en inhibant le complexe CDK4/cyclin D1 (Sherr et al., 2000). L'inactivation du gène P53 participe également à la croissance incontrôlée des cellules de GBM.

2. Mort cellulaire

Trois types de mort cellulaire se distinguent par leur mode de déclenchement et leur mode de fonctionnement (Hotchkiss et al., 2009).

- l'apoptose génétiquement programmée en cas de stress cellulaire ou de radiation
- la nécrose considérée comme une mort accidentelle
- l'autophagie déclenchée par les cellules pour survivre en cas de privation en nutriments ou en facteurs de croissance et pendant laquelle elles recyclent leurs propres organelles non essentiels, redondants ou endommagés, ainsi que des composants macromoléculaires.

Résistance à l'apoptose des cellules tumorales de GBM

Les caspases, de la famille des protéases, présentes sous forme constitutive et activées après leur clivage, sont les effecteurs centraux de l'apoptose. L'apoptose est déclenchée par une activation de la chaine des caspases. Cette activation s'effectue principalement par deux voies. La première est due à l'activation des membres de la famille du récepteur au TNF qui après leur oligomérisation suite à la fixation de leur ligand, conduit au recrutement de la protéine adaptatrice FADD qui interagit avec le prodomaine des caspases 8 ou 10, déclenchant ainsi leur activation. La deuxième voie

débute par le relargage sous l'effet d'un stress cellulaire du cytochrome c des mitochondries vers le cytosol, où il se lie à la protéine adaptatrice APAF-1 qui recrute et active alors les caspases 9 initiatrices pour former le complexe apoptosome. Les caspases 8 ou 10 et les apoptosomes activent ensuite les caspases 3 effectrices dont les substrats sont des protéines astructurelles, des protéines impliquées dans le cycle cellulaire ou dans la réparation de l'ADN, des kinases et des protéines de signalisation (Krakstad et al., 2010). L'action de la caspase 3 aboutit à la mort de la cellule.

L'activité des caspases est régulée par les protéines de la famille Bcl-2 au niveau mitochondrial et par les IAPs (Inhibitor of apoptosis proteins) qui inhibent les caspases en se liant à elles. Pendant les phases de réparation, quand les dommages sont trop importants, p53 induit l'apoptose. Dans les GBM, il y a une surexpression de la protéine anti-apoptotique Bcl-2, une mutation inactivant p53 entrainant donc une résistance à l'apoptose et une délétion de $p14^{ARF}$, protéine stabilisant p53, participant ainsi à la résistance à l'apoptose des cellules.

Survie des cellules tumorales

La signalisation de la survie cellulaire protège les cellules cancéreuses de la mort causée par des dommages létaux de l'ADN autres que ceux de l'apoptose, en induisant l'expression ou la disponibilité de facteurs de survie. Elle se distingue de la résistance à l'apoptose. NFkB (Nuclear factor kB) est un facteur de la transcription qui active l'expression de gènes impliqués dans la survie cellulaire mais aussi dans la migration et la prolifération (Karon et al., 2002).

L'activation d'Akt induit la survie des cellules néoplasiques des GBM en facilitant la translocation nucléaire du facteur NFkB et donc la transcription des gènes impliqués dans la survie cellulaire.

Présence de nécrose

La nécrose a lieu lorsqu'il y a une grande modification des conditions physiologiques. Elle est caractérisée par la perte de l'intégrité de la membrane plasmatique qui aboutit à un influx d'ions et de fluides extracellulaire. Il en résulte un gonflement caractéristique des cellules en nécrose ainsi que de leurs organelles et de l'échappement d'enzymes protéolytiques des lysosomes vers le cytosol. La membrane plasmique se rompt alors laissant se déverser le contenu du cytoplasme vers le milieu extracellulaire.

La nécrose (avec l'angiogenèse) est la caractéristique histologique spécifique des glioblastomes. Malgré le pouvoir angiogénique de ces tumeurs, des cellules sont en hypoxie, du fait de leur abondance causée par leur prolifération accrue et leur résistance à l'apoptose. En l'absence de nouvelle vascularisation suffisante dans ces zones pour leur fournir les apports énergétiques indispensables à leur survie, ces cellules en hypoxie finissent par se nécroser.

3. Invasivité des cellules tumorales de GBM

La plupart des cellules font preuve de motilité durant le développement embryonnaire, la cicatrisation ou les réponses immunitaires. La motilité des cellules est étroitement contrôlée sauf pour les cellules tumorales. Les cellules tumorales peuvent faire appel à trois types de migration (Yilmaz et al., 2010) :

- la migration individuelle mésenchymale. C'est le type de migration classique de la métastase.
- La migration individuelle amoeboïde
- La migration collective.

Les cellules tumorales des GBM utilisent le mode de migration individuelle mésenchymale (Zhong et al., 2010).

Certaines altérations génétiques induisent l'invasion cellulaire des GBM. L'amplification de l'activité du récepteur EGFR (Sieg et al., 2000) et la mutation

inhibitrice de PTEN (Tamura et al., 1998) induisent la migration des cellules, en agissant sur les adhésions focales. De même l'amplification de l'activité du récepteur PDGFR augmente la migration des cellules en même temps que leur prolifération.

Mais l'invasion des GBM est surtout due à l'activité autocrine et paracrine de facteurs secrétés par la tumeur elle-même (Hoelzinger et al., 2007).

Le microenvironnement participe au phénotype malin des tumeurs. Par définition, le microenvironnement tumoral regroupe la MEC et le stroma. La MEC constituant la lame basale, sépare normalement le parenchyme du stroma. Or dans le cancer l'intégrité de la lame basale est perdue. La fonction des cellules est conférée par leur interaction avec leur microenvironnement. Or les cellules saines du microenvironnement cérébral qui entourent la tumeur favorisent la migration des cellules tumorales en même temps que leur croissance et l'angiogenèse, accentuant le phénotype malin des GBM (Hoelzinger et al., 2007).

Linvasivité des GBM se caractérise en immunohistochimie par la présence de cellules tumorales isolées dans le parenchyme cérébral avoisinant la tumeur. En IRM ou scanner, elle se caractérise par un aspect général de la tumeur en « doigt de gant » caractéristique de son infiltration dans le tissu avoisinant.

Le caractère invasif est l'une des caractéristiques qui définit les GBM et est responsable de leur caractère agressif et de leur mauvais pronostic car ils récidivent systématiquement à proximité du site orignal de la tumeur (Lee SW et al., 1999) ou à distance du cerveau (van Nifterik et al., 2006) à cause de leur excision difficile et de la croissance des cellules invasives restantes qui n'ont pas été affectées par les traitements. Cependant, malgré cette infiltration extensive dans le tissu cérébral sain, les GBM métastasent rarement en dehors du cerveau (Zhong et al., 2010).

IV. Implication de l'angiogenèse dans la malignité des GBM

1. Implication de l'angiogenèse dans la malignité des GBM

La barrière hémato-encéphalique

Dans le cerveau en développement, les capillaires se différencient et murissent en barrière hémato-encéphalique (BHE). Elle représente l'interface et le lien entre le système nerveux central et la circulation sanguine. Elle a un rôle protecteur en bloquant et modulant le passage des molécules, en maintenant l'homéostasie ionique et en guidant les cellules immunes. Un rôle nutritif via la diffusion du sang vers le système nerveux central des ions, peptides et nutriments. Elle est présente dans toutes les régions du cerveau sauf dans les régions qui régulent le système nerveux autonome et les glandes endocrines du corps.

Etapes de l'angiogenèse tumorale

L'angiogenèse tumorale définit l'ensemble des processus cellulaires et moléculaires conduisant à la formation de nouveaux vaisseaux sanguins à partir d'un réseau vasculaire existant. L'hypoxie et les altérations génétiques des cellules tumorales des GBM induisent l'expression de facteurs angiogéniques par la tumeur, qui activent leurs récepteurs exprimés à la surface des cellules endothéliales des microvaisseaux alentours (Whong et al., 2009). L'angiogenèse est alors initiée.

Elle débute par l'augmentation de la perméabilité des microvaisseaux, menant à l'extravasation des protéines plasmatiques et à la dissociation de leur couverture péricytaire. Les cellules endothéliales peuvent alors proliférer et migrer après avoir dégradé la membrane basale et à mesure qu'elles réorganisent la MEC. La dégradation de la matrice nécessite l'expression par ces dernières de la cathepsine B et des enzymes protéolytiques telles que les métalloprotéases (MMPs). Les cellules endothéliales la reconstituent ensuite en exprimant la fibronectine, la laminine, la ténacine C et la

vitronectine. Le nouveau microvaisseau prend forme lorsque les cellules endothéliales s'alignent en mode bipolaire et s'organisent en tubule, formant ainsi la lumière vasculaire. Les différents « sprouts » ainsi formés le long du vaisseau préexistants se connectent entre eux, formant des boucles vasculaires à l'intérieur desquelles le sang se met alors à circuler. Les microvaisseaux nouvellement formés deviennent stables et matures après le recrutement de péricytes et la reconstitution de la lame basale.

« switch angiogénique » : facteurs pro- et anti-angiogéniques

L'homéostasie vasculaire est gouvernée par un équilibre entre les facteurs pro- et anti-angiogéniques.

Les facteurs anti-angiogéniques sont de quatre groupes : les interférons (IFNα, IFNβ et IFNγ), les interleukines (IL-4, IL-10 et IL-12), les inhibiteurs de métalloprotéases (TIMP1, TIMP2 et TIMP3) et des fragments protéolytiques. Les facteurs pro-angiogéniques sécrétés par les GBM sont les facteurs de croissance VEGF, FGF, PDGFb, Agiopoiétines, EGF, TGFα, TGFβ mais aussi SF/HGF, CYR61, CTDF, IGF-1, les interleukines IL-6, IL-8, TNFα et les métalloprotéases MMP2 et MMP9.

L'hypoxie augmente l'expression des facteurs pro-angiogéniques par les cellules tumorales via l'activité transcriptionnelle de HIF1α. C'est particulièrement le cas pour VEGFa que l'on retrouve pour cette raison très exprimé par les cellules palissadiques autour des zones nécrosées. Les mutations propres aux GBM telles que la mutation inhibitrice de PTEN et la mutation activatrice de EGFRvIII augmentent également l'expression du VEGF par voie transcriptionnelle dans les GBM.

L'activité de l'ensemble des facteurs pro-angiogéniques conduit à une forte angiogenèse en activant différentes étapes.

2. Description de la vascularisation des GBM

La structure et la fonctionnalité des vaisseaux des GBM sont anormales. Les vaisseaux néoformés au sein des GBM présentent des caractéristiques structurales spécifiques : paroi épaissie en raison de l'hyperplasie des cellules endothéliales, la perméabilité vasculaire est augmentée et la lame basale est plus fine (Vajkoczy et Menger, 2000).

Le grand pouvoir angiogénique des GBM se traduit à l'échelle microscopique par un réseau vasculaire anormal constitué de vaisseaux proliférant dont les cellules sont fréquemment en mitose et expriment des marqueurs de prolifération. L'intensité de la prolifération vasculaire est spécifique des GBM et se présente sous deux formes distinctes : une augmentation diffuse de la densité vasculaire avec des petits vaisseaux et le forme complexe connue sous le terme de prolifération microvasculaire (Lousi DN, 2006).

La structure anormale des microvaisseaux des GBM entrave la délivrance des chimiothérapies et augmente l'hypoxie. De plus, elle crée une niche vasculaire qui abrite des cellules souches qui constituent une partie de la tumeur et participent à la résistance aux traitements. La forte vascularisation observée dans les GBM est également en partie responsable d'œdème péritumoral qui résulte de la barrière hémato-encéphalique défectueuse des nouveaux vaisseaux (Vaquero et al., 2000).

V. Caractéristiques et traitement des glioblastomes

1. Caractéristiques en imagerie

Un effet de masse, visible en IRM, avec une zone nécrotique et des parois irrégulières prenant fortement le contraste peuvent suggérer le diagnostic (Figure 1). Une scanographie montre en général une masse non homogène avec un centre hyperdense étendu en un anneau de taille variable entouré d'œdème.

Comme de multiples pathologies peuvent avoir parfois le même aspect radiologique et que le GBM peut avoir différents aspects, le diagnostic définitif est généralement effectué lors de l'examen histologique.

2. Caractéristiques en histologie

Les GBM sont caractérisés par la présence de zones de tissus nécrosés entourées de cellules hautement anaplasiques. Les GBM étant des tumeurs très infiltrantes, on retrouve à leur périphérie des cellules tumorales isolées infiltrant le parenchyme adjacent, caractérisées par des noyaux atypiques allongés.

Ces tumeurs présentent une très forte densité cellulaire associée à des atypies nucléaires et un index mitotique élevé. On observe également une néoangiogenèse intense et une prolifération endothéliocapillaire caractéristique. Les plages de nécrose et les palissades périnécrotiques font partie des caractéristiques histologiques des GBM et correspondent a des régions souffrant d'hypoxie (Figure 2).

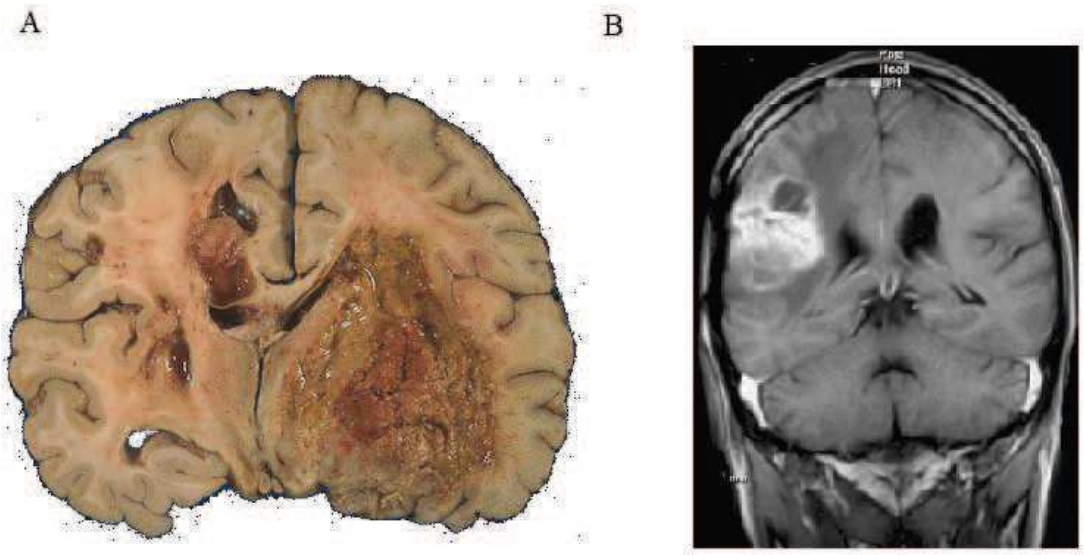

Figure 1 : A/ Aspect macroscopique d'un GBM. B/ Coupe coronale du cerveau, IRM pondérée T1 après injection de produit de contraste.

D'après A/ The internet pathology laboratory. B/ info-radiology.ch.

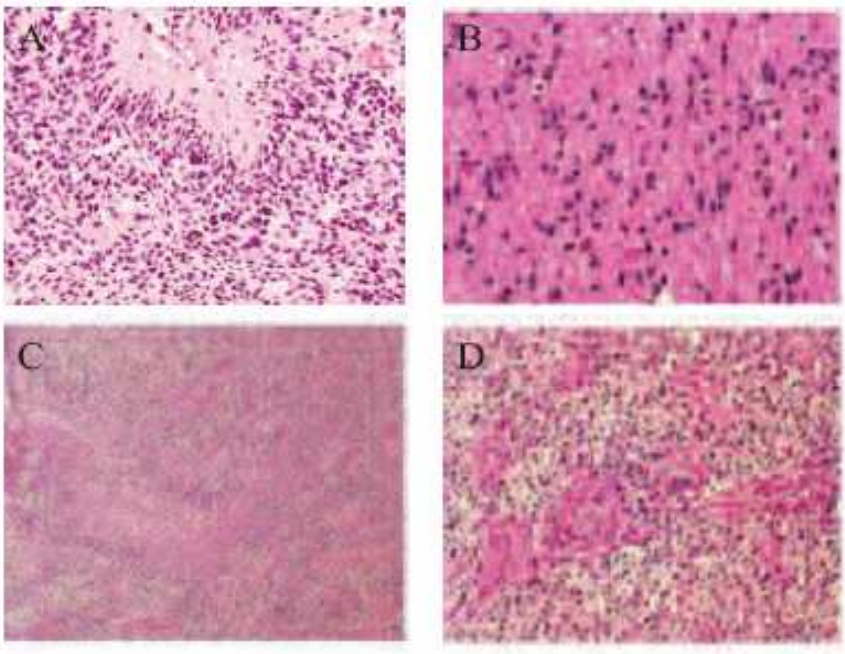

Figure 2: Aspects histologiques et macroscopiques d'un GBM. A : plage de nécrose ; B : parenchyme cérébral infiltré par des cellules tumorales isolées ; C et D : palissades périnécrotiques et prolifération microvasculaire.

D'après Figarella-Branger et al., 2008.

3. Traitement des glioblastomes

Le traitement standard actuel du GBM nouvellement diagnostiqué est la résection chirurgicale suivie d'une radiochimiothérapie concomitante suivie d'une chimiothérapie adjuvante au Témozolomide (Témodal [@]), selon le protocole de Stupp (Stupp et al., 2005).

a. Neurochirurgie

L'exérèse est toujours considérée comme le principal élément du traitement. Plusieurs statistiques (Mondor, Freiburg, Protocole Stupp de 2005) confirment une amélioration de la médiane de survie en cas d'exérèse totale. Il peut aussi y avoir également des zones résiduelles et parfois des récidives ou des tumeurs bénignes peuvent récidiver en tumeur maligne (anaplasique). Il convient dont très souvent après contrôle de traiter ces tumeurs par radiothérapie pour éviter la récidive.

b. Radiothérapie

La radiothérapie est en général plus efficace que la chimiothérapie mais 20% des GBM sont radiorésistants. La radiosensibilité des tumeurs hypoxiques, et surtout l'augmentation de la dose initiale d'irradiation délivrée n'ont pas permis d'améliorer les résultats du traitement de ces tumeurs. La chimiothérapie concomitante vient de remporter un nouveau succès dans le traitement des GBM. L'adjonction de Témozolomide pendant toute la durée de l'irradiation, suivie d'une administration adjuvante, permet une amélioration significative de la probabilité de survie, au prix d'une majoration minimale de la toxicité immédiate.

c. Chimiothérapie

Les agents akylants et les nitro-urées (BCNU, Camustine[@]) sont les drogues les plus utilisées en chimiothérapie. Le Temodal[@] (Témozolomide), agent alkylant, est le premier agent chimiothérapeutique oral qui traverse facilement la barrière

hématoméningée. Ces traitements permettent d'augmenter la survie globale des patients généralement de quelques mois, même chez les sujets âgés (Keime-Guibert et al., 2007). Cependant, compte-tenu du pronostic très sombre des GBM et de leur caractère angiogénique et invasif, de nombreux essais thérapeutiques sont en cours notamment autour de la thérapie anti-angiogénique.

Les GBM sont des tumeurs très vascularisées dans lesquelles le concept établi par Folkman d'une relation étroite entre croissance tumorale et néoangiogenèse s'est vérifié.

Le laboratoire a montré que l'expression du peptide Adrénomédulline était corrélée au grade de malignité des gliomes, les glioblastomes exprimant donc le plus d'AM (Ouafik et al., 2002). De nombreuses études alors entreprises sur le rôle de l'Adrénomédulline dans les glioblastomes ont mis en évidence une implication de ce peptide dans leur croissance. Dans une seconde partie, nous présenterons l'Adrénomédulline ainsi que son mode d'action et ses effets biologiques.

PARTIE 2 : L'Adrénomédulline et son récepteur

I. L'adrénomédulline (AM)

L'adrénomédulline (AM) est un peptide qui fut isolé par Kitamura en 1993 à partir de phéochromocytome humain (Kitamura et al., 1993). Du fait de leur homologie de séquences, l'AM appartient à la famille des peptides apparentés à la Calcitonine, parmi lesquels on trouve la calcitonine, l'amyline et le CGRP (Calcitonin Calcitonin Related Peptide). Dés sa découverte, l'AM a suscité beaucoup d'intérêt pour la recherche cardiovasculaire du fait de son fort effet hypotenseur causé par la dilatation des vaisseaux. Depuis de nombreux autres effets biologiques de l'AM ont été démontrés et ce, sur de nombreux autres types cellulaires en conditions physiologiques et pathologiques.

1. Biosynthèse

Le gène de l'AM est localisé sur le locus P15.1-3 du chromosome 11 chez l'homme et est constitué de 4 exons et 3 introns (Ishimitsu et al., 2001). Le clonage de l'ADNc codant pour l'AM a révélé que l'AM est générée à partir d'un précurseur, la préproamédulline (préproAM) constitué de 185 acides aminés (Kitamura et al., 1993). En plus de l'AM en C-ter, la préproAM contient en N-ter un autre peptide bioactif, la proAM de 20 acides aminés (PAMP :N-terminal ProAM 20 Peptide). Ce peptide abaisse la pression sanguine lorsqu'on l'injecte en intraveineuse mais son action est plus large que celle de l'AM et il y a assez peu d'informations sur son rôle dans la vascularisation.

Dans une analyse de la séquence de l'ADN génomique de la préproAM, il a été démontré que l'AM était encodée dans le 4ème exon et PAMP dans les 2ème et 3ème exons

(Ishimitsu et al., 1994). Au sein de la préproAM, les segments Gly-Lys-Arg (GKR) et Gly-Arg-Arg (GRR) sont directement adjacents à la séquence de PAMP et de l'AM respectivement. Le clivage de la préproAM par des endoprotéases, donne naissance à la forme intermédiaire inactive de l'AM (iAM) et de la PAM (iPAMP), toutes deux présentant en C-ter le résidu Gly. L'ajout d'une fonction amide sur l'acide carboxylique libre dans une réaction catalysée par l'enzyme d'amidation PAM (peptidyl-glycine alpha-amidating monooxigenase), convertit iAM et iPAMP en leur forme mature (mAM et mPAMP) possédant alors une structure amide en C-ter (Kitamura et al., 1998) (Figure 3).

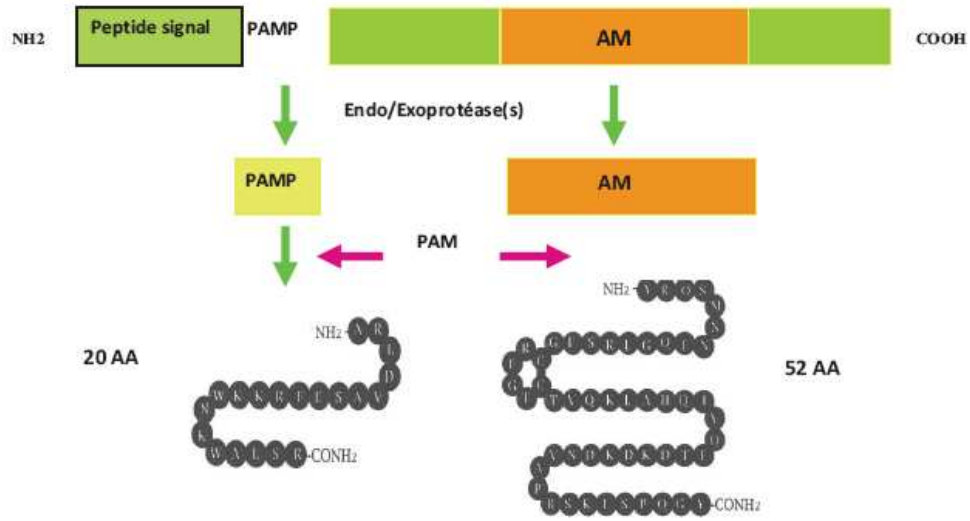

Figure 3 : Biosynthèse de l'AM et de la PAMP

2. Structure

L'AM est un peptide de 52 acides aminés comportant une tyrosine amidée en C-ter et une boucle de 6 acides aminés (16-21) formée par un pont disulfure entre les résidus Cys 16 et Cys 21. Cette structure en anneau et le résidu amide en C-ter sont tous deux essentiels pour sa liaison à son récepteur et son activité biologique (Kitamura et al., 2002).

En effet, l'étude de la relation structure/activité de l'AM a été réalisée en utilisant des analogues synthétiques de l'AM dans des cellules vasculaires musculaires lisses (vSMC) de rat (Eguchi et al., 1994) et en observant l'activité de l'adénylate cyclase. Les deux dérivés tronqués en N-ter, le hAM-(13-52)-NH_2 et le hAM-(16-52)-NH_2 présentent des Ki et une activité de l'AMPc similaires à ceux de la forme complètement mature de l'AM humaine hAM-(2-52)-NH_2. Le fragment N-ter seul non amidé hAM-(1-10)-OH n'ayant lui aucun effet. Le fragment N-ter n'est donc pas nécessaire pour l'interaction de l'AM avec son récepteur. Au contraire, la suppression de l'amidation en C-ter résulte en une diminution significative de l'activité de l'AMPc. De plus, le clivage du pont disulfure entre les Cys 16 et 21 par carbométhylation, résulte en une suppression de la réponse AMPc. Ces données indiquent donc que l'amidation C-ter de l'AM et la structure en anneau de l'AM jouent un rôle fondamental dans son interaction avec son récepteur et la réponse AMPc.

3. Régulation

Il est désormais reconnu que toute cellule est susceptible d'exprimer et secréter de l'AM (Isumi et al., 1998a ; Tomoda et al., 2001b ; Minamino et al. 2002).

Dans les cellules de type endocrine, l'AM stockée dans les granules est sécrétée immédiatement après stimulation. Dans les cellules non endocrines, l'expression et la sécrétion de l'AM augmentent au bout d'une à deux heures après stimulation.

L'hypoxie, les hormones et les molécules pro-inflammatoires sont les principaux facteurs influençant l'expression et la sécrétion de l'AM.

Hypoxie et stress oxydatif

L'hypoxie est un stimulant important de l'expression de sécrétion de l'AM. Cinq éléments de réponse HRE (Hypoxia-Responsive Element) sont d'ailleurs localisés au niveau des nucléotides 1095 et 770 dans la région promotrice du gène de l'AM (Zudaire et al., 2003). De même la région 5' du gène humain de l'AM présente un site consensus de fixation pour le facteur HIF-1 (Garayoa et al., 2000). Ainsi une augmentation de l'expression du gène ainsi que de la sécrétion de l'AM sous hypoxie ont été démontrées dans les cellules endothéliales de la veine ombilicale humaine, HUVECs (Ogita et al., 2001) et dans les cellules épithéliales de la rétine (Udono et al., 2001). Les facteurs NF-kα et β (Tumor Necrosis Factor) activés sous hypoxie, augment l'expression et la production d'AM (Sugo et al., 1994 ; Yoshihara et al., 2002). Il a été également rapporté qu'un facteur de transcription impliqué dans l'induction de l'expression des gènes régulés par l'hypoxie, la protéine EPAS1 (endothelial PAS domain protein 1) induisait l'expression du gène de l'AM (Tanaka et al., 2002).

Il a été démontré que le stress oxydatif régule également l'expression de l'AM (Ando et al., 1998). En plus de l'hypoxie, des études ont montré que l'hémorragie et le choc septique augmentaient l'expression du gène de l'AM dans divers types cellulaires et tissus ainsi que la concentration plasmatiques (Yashibayashi et al., 1999 ; Ueda et al., 1999).

Cytokines pro-inflammatoires et LPS

Des patients présentant des infections ou des troubles inflammatoires possèdent des concentrations plasmatiques élevées d'AM (Ishimitsu et al., 1994 ; Hirata et al., 1996). Le peptide aurait pour rôle dans ces circonstances de dilater les vaisseaux sanguins et de

faciliter la circulation du sang et des leucocytes dans les tissus enflammés. La production d'AM serait augmentée après stimulation par des cytokines telles que l'IL-1 (interleukine -1) et le TNF-α tumor necrosis factor-α). L'INF-γ supprime l'expression du gène et la sécrétion d'AM dans les cellules épithéliales alors qu'il le stimule dans les monocytes et macrophages.

Chez le rat, l'administration de LPS (endotoxine exprimée à la surface membranaires des bactéries Gram-) pendant trois heures provoque une augmentation de l'expression du gène de l'AM de 2 à 7 fois (Shoji et al., 1995 ; Chent et al., 2004 ; Cheung et al., 2004).

Facteurs de croissances et hormones

Les facteurs de croissance TGFβ, FGGFb et EGF peuvent diminuer l'expression de l'AM. L'angiotensine II et l'endotheline-1 peuvent l'augmenter (Sugo et al., 1995).

Les hormones stéroïdiennes affectent la production d'AM.

Autres facteurs

L'AM elle-même peut indirectement réguler sa propre expression.

Stress physique

Plusieurs groupes ont montré que le niveau d'AM plasmatique était augmenté lors d'une hypertension (Ishimitsu et al. 1994 ; Kato et al., 1999).

II. Le récepteur de l'adrénomédulline

L'AM exerce son action biologique à travers deux récepteurs à la surface membranaire. Chacun de ces récepteurs est formé d'un complexe multi protéique composé de deux éléments (McLatchie et al., 1998) : le CLR (Calcitonin receptor like receptor) et l'une des molécules associées appelées RAMPs (Receptor activity-modifying protein).

1. CLR

Le CLR nommé ainsi de part sa similitude avec le récepteur de la calcitonine, fut identifié en 1993 (Njuki et al., 1993 ; Chang et al., 1993). C'est une protéine à 7 domaines transmembranaires appartenant à la classe B des récepteurs couplés aux protéines G (RCPG). Il est donc de nature polypeptidique et comporte une partie extracellulaire N-ter possédant le site de liaison du ligand, une partie transmembranaire à 7 hélices et une partie intracellulaire C-ter en contact avec la protéine G, qui assure le transfert et l'amplification du signal reçu par le récepteur. Le CLR présente également 3 boucles extracellulaires, 3 boucles intracellulaires (Bockaert et Pin, 1999) et il peut être sujet à trois N-glycosylation post-traductionnelles différentes dans le domaine N-ter.

2. RAMP

Les RAMPs (Receptor activity-modifying protein) sont des protéines à un domaine transmembranaire et il en existe trois nommées RAMP1, RAMP2 et RAMP3. Elles possèdent un long domaine N-ter extracellulaire et un domaine intracellulaire C-ter court. Les trois RAMPs partagent une structure de base similaire qui inclut quatre cystéines conservées en N-ter, sans doute importantes pour la structure secondaire de cette région de la protéine. Chez l'homme, RAMP1 présente 148 acides aminés et deux sites de phosphorylation du côté C-ter, tout comme RAMP2 et RAMP3. Cependant, RAMP2 présente 191 acides aminés et un site de N-glycosylation alors que RAMP3 est constitué de 148 acides aminés et possède quatre sites de N-glycosylation.

3. Les complexes CLR/RAMP

Les complexes CLR/RAMP2 et CLR/RAMP3 constituent à la surface membranaire les deux récepteurs de l'AM. Le complexe CLR/RAMP1 constitue le récepteur du CGRP. Les protéines RAMPs jouent un rôle fondamental dans la détermination de la spécificité

du récepteur CLR et du récepteur à la calcitonine (CTR) ainsi que dans leur fonctionnalité.

En effet, le CLR nécessite son interaction avec l'une des trois RAMPs pour être transloqué du compartiment intracellulaire vers la surface membranaire et cela est réciproque pour les RAMPs (Figure 4). L'identité de la protéine RAMP qui se lie à ces récepteurs couplés aux protéines G détermine la spécificité du récepteur. Cependant, il n'est pas encore clair, si les RAMPs définissent la spécificité des ligands en participant directement à la constitution du site de liaison ou bien en provoquant une modulation allostérique de la conformation du récepteur en s'y liant.

4. Régulation de l'expression de CLR, RAMP2 et RAMP3

Le clonage de la région promotrice du CLR par Nikitenko et al., et la découverte de la présence de la zone HRE dans celle-ci a permis de mettre en évidence, au sein des cellules endothéliales microvasculaires transfectées, que HIF-1 activait le promoteur ainsi que la transcription du gène du CLR en se liant à son promoteur.

A l'inverse, dans les cellules vasculaires du muscle strié squelettique, une hypoxie chronique induit une surexpression protéique des trois RAMPs et du CLR (Cueille et al., 2005).

5. Régulation de l'activité du système AM/AM-R

L'AM a une demi-vie de 22 minutes (Meeran et al., 1997), ce qui rend son dosage plasmatique difficile. Une protéine sérique liant l'AM, l'AMBP-1 (Adrenomedullin binding protein-1) régule la biodisponibilité de l'AM (Elsasser et al., 1999) et a été décrite et caractérisée comme étant le facteur H du complément humain (Pio et al., 2001 ; Zudaire et al., 2003a). De manière générale, les protéines de liaison limitent le transport du peptide dans l'espace interstitiel et l'accès à ses récepteurs spécifiques. Elles modulent ainsi l'activité biologique du peptide et le protègent contre la clairance

métabolique par les protéases, prolongeant sa demi-vie dans la circulation (Martinez et al., 2001).

L'internalisation des récepteurs de l'AM peut constituer un mode de régulation de l'activité du récepteur. En effet, chacun des trois couples CLR/RAMP est internalisé de façon chlatrine dépendante après stimulation agoniste par leur ligand respectif et est majoritairement destiné à une dégradation lysosomale plutôt qu'à un recyclage membranaire (Kuwasako et al., 2000). Les complexes CLR/RamP sont d'ailleurs très stables et maintenus pendant l'internalisation autocrine du récepteur.

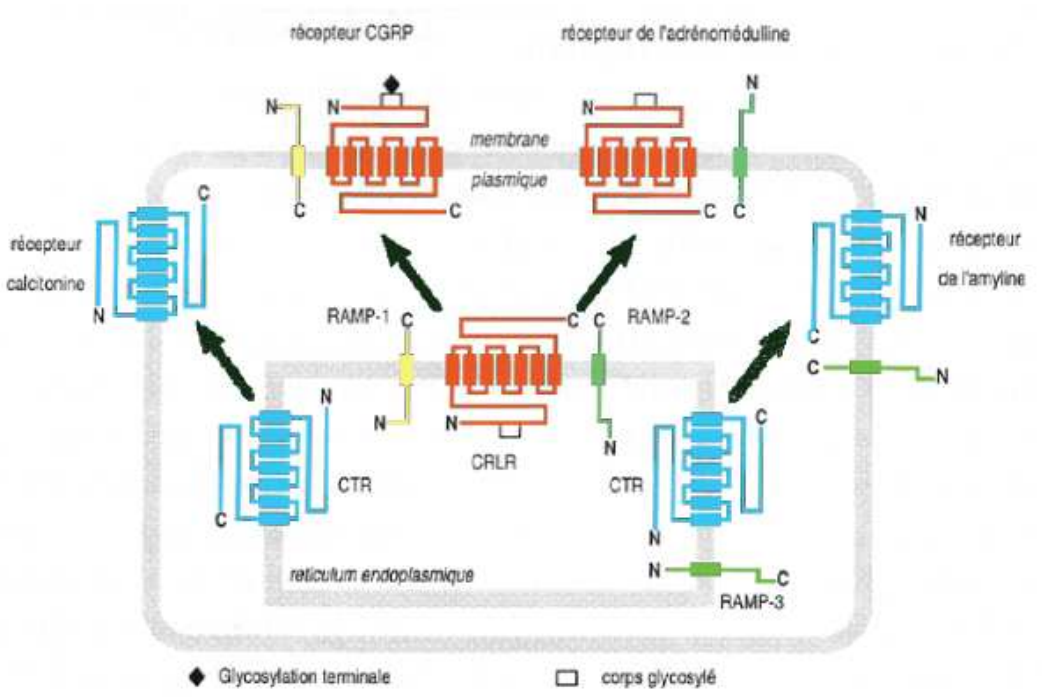

Figure 4 : Rôle des protéines RAMPs dans l'identité finale des récepteurs de la famille de la calcitonine

III. Action biologique de l' adrénomédulline

1. Expression dans l'organisme

Expression tissulaire de l'AM

Chez les mammifères, la distribution tissulaire de l'AM est ubiquitaire mais une plus forte expression est observée dans le système cardiovasculaire, le rein, le poumon, le cerveau et la glande surrénale (Kitamura et al., 1993a, Ichiki et al., 1994 ; Hinson et al., 2000).

L'AM est aussi retrouvée dans le plasma sanguin et lymphatique. Ses concentrations plasmatiques sont d'ailleurs augmentées dans certaines pathologies comme l'hypertension artérielle, l'insuffisance cardiaque et rénale, le diabète et les chocs septiques (Miller et al., 1996 ; Kitamura et al., 1997 ; Martinez et al., 2002 ; Oehler et al., 2002).

Expression tissulaire du CLR, RAMP2 et RAMP3

Le CLR est exprimé dans divers tissus chez l'homme et le rat (Aiyar et al., 1996 ; Njuki et al., 1993 ; Chakravarty et al., 2000) mais son expression est prédominante dans les vaisseaux sanguins des organes (Nikitenko et al., 2001 ; Oliver et al., 2002 ; Hagner et al., 2002).

Les RAMPs sont largement exprimés dans les tissus et lignées cellulaires (cœur, cerveau, pancréas, poumons, reins, trachée, cellules endothéliales, cellules du système immunitaire) (Chakravarty et al., 2000 ; Owji et al., 1995).

2. Action dans l'organisme

A l'origine, la puissante action vasodilatatrice de l'AM a été décrite et ses actions biologiques avaient donc été principalement focalisées sur le système cardiovasculaire. Depuis, de nombreuses études ont révélé un grand champ d'action de l'AM dans le

système nerveux central, le système endocrinien, digestif, reproductif, immunitaire ainsi que dans divers organes et tissus dans lesquels elle est exprimée avec ses récepteurs.

Action dans le système cardiovasculaire

Des effets hypotenseurs de l'AM furent d'abord mis en évidence (Kitamura et al., 1993a). Cette réduction de la pression sanguine était associée à une diminution de la résistance périphérique, sans altérer le rythme cardiaque.

L'effet vasodilatateur de l'AM a été décrit dans la vascularisation systémique mais aussi dans les vaisseaux rénaux (Hirata et al., 1995), pulmonaires (Lippton et al., 1994), cérébraux (Lang et al., 1997) et dans la circulation coronaire (Yoshimoto et al., 1998). Cet effet est issu d'au moins deux mécanismes distincts. Le premier est l'action directe sur les cellules musculaires lisses dans lesquelles l'AM élève le taux d'AMPc (Eguchi et al., 1994) en agissant via le récepteur du CGRP (Nuki et al., 1993). La deuxième est l'action sur les cellules endothéliales via ses propres récepteurs. L'AM induit de façon dose dépendante une augmentation de l'AMPc intracellulaire et une augmentation du Ca^{2+} intracellulaire suite à l'activation de la phospholipase C et la formation de l'IP_3 (Inositol 1,4,5-trisphosphate) qui cause à son tour l'activation de Nos (Nitric Oxide synthase) (shimekake et al. 1995).

En physiopathologie cardiaque, l'augmentation de l'AM est fréquemment observée notamment après ischémie (Miyashita et al. 2006). Par ailleurs l'AM semble avoir de nombreuses applications cardioprotectrices dans le traitement des pathologies cardiovasculaires (Okumura et al., 2003 ; Ishimitsu et al., 2006).

Action dans le système nerveux central

L'AM est détectée dans les astrocytes (Takahashi et al., 200a) ainsi que dans les neurones de la moelle épinière et du cerveau, en particulier dans le thalamus, l'hypothalamus et l'hypophyse.

L'une des fonctions hypothalamiques est le contrôle du comportement alimentaire et l'AM a été montrée impliquée de façon importante dans la régulation de la prise d'eau et de nourriture.

Chez des rats conscients, l'injection centrale d'M augmente le débit urinaire et l'excrétion de sodium et de potassium dans les urines, indiquant que des actions centrales de l'AM correspondent avec ses effets périphériques. L'action centrale de l'AM sur la pression sanguine systémique montre cependant un contraste frappant avec l'effet hypotenseur de l'AM à la périphérie. L'administration centrale d'AM augmente la pression sanguine et la fréquence cardiaque chez les rats non restreints anesthésiés (Takahashi et al., 1994) et conscients (Saita et al., 1998).

Action sur les reins

L'expression de l'AM et de ses récepteurs dans les différents compartiments du rein suggérait que l'AM était impliquée dans la régulation de l'hémodynamique rénale, la filtration glomérulaire et l'homéostasie du Na^+ tubulaire *in vivo*.

Action dans le système endocrinien

L'AM est détectée dans l'axe hypothalamo-hypophysaire, elle est supposée moduler la sécrétion des hormones hypophysaires et surrénales. C'est notamment le cas pour l'ACTH dont la sécrétion est inhibée par l'AM injectée en intraveineuse (Samson et al., 1995) et augmenté par l'AM injectée en intracérébroventriculaire (Shan et Krukoff, 2001). L'AM influence également la sécrétion de l'ocytocine qu'elle stimule (Serino et al., 1999) et la vasopressine qu'elle diminue (Yokoi et al., 1996).

3. Action sur la prolifération

L'AM peut avoir un effet stimulateur ou inhibiteur sur la prolifération cellulaire en fonction du type cellulaire. Mais le double effet de l'AM sur la prolifération cellulaire

est également dû à l'état de quiescence des cellules. L'AM exercerait son action mitogénique dans les cellules quiescentes via l'activation de la voie des MAPK.

4. Action sur l'apoptose

Un effet anti-apoptotique de l'AM a été observé sur divers types cellulaires entre autres les cellules glomérulaires , les kératinocytes, les fibroblastes et les cellules endothéliales. Cette action s'effectue indépendamment de la voie AMPc/PKA. L'AM entraine une surexpression du gène Max qui bloque l'apoptose induite pas le proto-oncogène Myc (Shichiri et al., 1999).

A l'inverse l'AM a un effet pro-apoptotique sur les cellules glomérulaires mésangliales via l'activation de l'AMPc/PKA (Parameswaran et al., 1999a) et également via la P38 MAPK.

5. Action sur la migration

Les études démontraient que l'AM inhibait la migration induite par le sérum, l'angiotensine II ou le PGFG (Horio et al., 1995 ; Kohno et al., 1997 ; Kohno et al., 1999) et ce via la voie de l'AMPc/PKA.

Cependant l'AM peut avoir un effet inverse en activant la migration des cellules en particulier des astrocytes.

L'AM est donc un peptide exprimé de façon ubiquitaire chez l'homme et a de ce fait un large champ d'action à travers l'organisme. Il agit de façon autocrine et paracrine via ses récepteurs CLR/RAMP2 et CLR/RAMP3 et est comparé aux facteurs de croissance du fait de ses effets sur la prolifération et la survie cellulaire pour lesquels l'AM peut présenter des effets opposés. Son expression et son activité étant particulièrement activées dans la vascularisation et lors de diverses pathologies, nous évoquerons dans la prochaine partie l'implication de l'AM dans les cancers et en particulier dans les glioblastomes multiformes.

PARTIE 3 : Rôle de l'Adrénomédulline dans la croissance tumorale des glioblastomes

Depuis la mise en évidence de l'AM au sein d'une tumeur de la médullo-surrénale humaine (Kitamura et al., 1993), plusieurs études ont montré l'expression de l'AM dans une variété de tumeurs et lignées cellulaires tumorales et ont décrit l'AM comme un maillon important dans la carcinogenèse et la progression tumorale dans divers cancers (Miller et al., 1996 ; Rocchi et al., 2001 ; Ouafik et al., 2002).

I.L'Adrénomédulline et hypoxie

L'hypoxie représente un facteur majeur d'induction de l'AM comme l'attestent les résultats qui associent une forte expression dans les cellules en palissade autour des zones de nécrose au sein des GBM, et les mécanismes de régulation de l'expression du gène de l'AM sous hypoxie (Garayoa et al., 2000 ; Ouafik et al., 2002). En effet, par hybridation *in situ*, il a été démontré *in vivo* qu'une forte expression de l'AM est associée aux zones de nécrose dans les GBM, suggérant que l'expression de l'AM dans ces tumeurs peut être induite par l'hypoxie (Boudouresque et al., 2005, Ouafik et al., 2002)

II.L'Adrénomédulline et contrôle du cycle cellulaire

1. Inhibition de la prolifération de lignées tumorales U87 in vitro

Des études ont montré que la neutralisation de l'action de l'AM sécrétée par les cellules tumorales in vitro conduit à l'inhibition très significative de la prolifération tumorale (Ouafik et al., 2002).

2. Effet de l'AM sur l'apoptose

La croissance tumorale peut être le résultat d'une prolifération cellulaire associée ou non à la mort cellulaire. L'AM induit la croissance tumorale en favorisant la prolifération cellulaire (Martinez et al., 2002) mais peut inhiber l'apoptose dans plusieurs types de tumeurs. Différentes études impliquent directement l'AM comme facteur anti-apoptotique des cellules en apoptose. L'AM réduit l'apoptose des cellules endothéliales de rat maintenues en culture dans un milieu sans hormones via un mécanisme AMPc dépendant (Kato et al. 1997). Cette abrogation de l'apoptose est due à une augmentation de l'expression du gène Max par l'AM par voie autocrine/paracrine (Shichiri et al., 1999). La protéine Max est un partenaire hétérodimérique du protooncogène c-myc lequel a un rôle dans la prolifération, la transformation et l'apoptose des cellules. Dans les cellules endothéliales quiescentes du rat, l'AM stimule l'expression de Max sans affecter l'expression de c-myc. Ainsi l'utilisation d'un anticorps anti-AM dans ces cellules maintenues en milieu sans hormones réduit le taux de Max et restaure le phénomène d'apoptose.

L'AM a une action protectrice sur les SMC en diminuant la production de ROS (reactive ocygen species) par un mécanisme APMc/PKA dépendant (Yoshimoto et al., 2004). Les ROS sont considérés comme des molécules toxiques à l'origine d'un stress oxydatif conduisant à la mort cellulaire. C'est une des causes de lésions des organes dans de nombreuses conditions pathologiques. L'expression de l'AM augmentée par le stress oxydatif empêche l'apparition de l'apoptose. L'AM serait un antioxydant intrinsèque.

III. Adrénomédulline et microenvironnement tumoral

Plusieurs équipes décrivent l'expression de l'AM et/ou de son récepteur CLR/RAMP dans la plupart des types cellulaires du microenvironnement tumoral. Ceci implique la reconnaissance par ces divers types cellulaires du signal AM et éventuellement des effets dans leur migration et leur prolifération.

1. AM et cellules endothéliales

Les cellules endothéliales constituent le 2^{éme} type cellulaire prépondérant du microenvironnement tumoral. L'AM est synthétisée dans les cellules endothéliales de la paroi vasculaire avec des taux 20 à 40 fois plus importants que ceux retrouvés dans les glandes surrénales (Sugo et al., 1994).

2. AM et péricytes

Plusieurs équipes ont montré l'expression d'AM dans les péricytes (Kis et al., 2002 ; Sugo et al., 1994). Les péricytes cérébraux sont connus pour embrasser la surface abluminale des vaisseaux. Leur fonction de soutien nécessitant une certaine plasticité de ces cellules, une étude récente a monté l'implication de l'AM dans la relaxation des péricytes cérébraux (Takata et al., 2009) via la voir PKA/AMPc.

3. AM, Macrophages et fibroblastes

L'expression et la sécrétion d'AM par les macrophages et d'autres cellules du système immunitaire est bien décrite dans la littérature (Kubo et al., 1998 Balasch et al., 2004). Plusieurs études ont montré la production d'AM par les fibroblastes (Isumi et al., 1998), l'AM induirait l'activité MMP-2 suggérant un rôle important dans la dégradation de la matrice extracellulaire.

IV.AM et contrôle de la densité vasculaire tumorale

L'inhibition de la croissance tumorale par un antagoniste des récepteurs de l'AM ou par un anticorps neutralisant le peptide est associée à une réduction de la densité des vaisseaux sanguins (Ouafik et al., 2002). En effet plusieurs études montrent la disparition de l'expression des marqueurs endothéliaux en immunohistochimie (Miseki et al., 2006).

Ceci suggère un rôle fondamental de l'AM dans la mise en place d'une vascularisation stable et fonctionnelle nécessaire à la croissance tumorale.

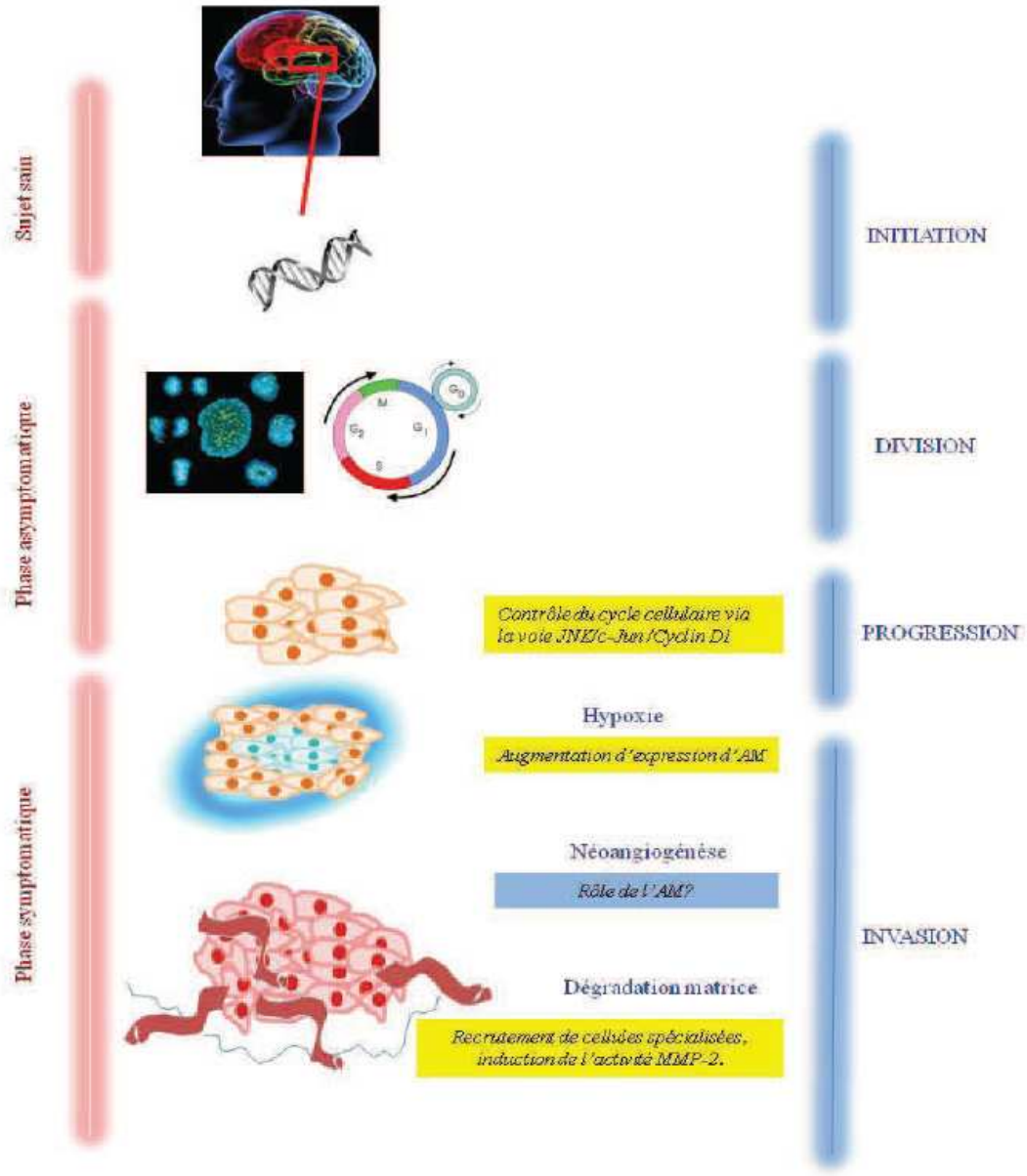

Figure 32 : Implication de l'AM dans le développement tumoral du GBM.

Figure 5 : Implication de l'AM dans le développement tumoral des GBM

PROBLEMATIQUE

Comme nous l'avons vu, l'AM est donc un facteur de croissance angiogénique impliqué dans la progression tumorale des glioblastomes. Exprimée par la composante tumorale en hypoxie mais également par la composante vasculaire, l'AM participe de façon autocrine et paracrine au développement de ces tumeurs.

Le but de ce travail sera dans un premier temps de mieux appréhender le rôle de l'AM dans l'angiogenèse des glioblastomes humains et de comprendre en quoi cette angiogenèse est différente de celle de tumeurs extrêmement angiogéniques mais bénignes comme les astrocytomes pilocytiques.

Un second aspect de ce travail sera d'étudier l'hypothèse selon laquelle le ciblage du système AM/récepteur (AMR) entrainerait une inhibition de l'angiogenèse et une suppression de la croissance tumorale dans un modèle de xénogreffe de glioblastome. En effet, un premier travail avait déjà démontré l'efficacité du ciblage de l'AM dans l'inhibition de la prolifération cellulaire in vitro et de la croissance tumorale in vivo.

La dernière partie de ce travail sera dans un premier temps de mettre en évidence l'existence du système AM/AMR dans les cellules gliales mais aussi dans les cellules endothéliales issues de glioblastomes humains opérés. Dans un second temps sera étudiée, la capacité de l'AM à induire la migration et l'invasion de ces deux types cellulaires et sa capacité à induire la formation de néovaissaux à partir des cellules endothéliales isolées.

PRESENTATION DES RESULTATS

PUBLICATION 1

Adrenomedullin Expression and Regulation in Human Glioblastoma, Cultured Human Glioblastoma Cell Lines and Pilocytic Astrocytoma.

Philippe Metellus[1,2*], Brigitte Voutsinos-Porche[1*], Isabelle Nanni-Metellus[3], Carole Colin[1], Frédéric Fina[3], Caroline Berenguer[1], Nadège Dussault[1], Françoise Boudouresque[1], Anderson Loundou[4], Dominique Intagliata[1], Olivier Chinot[3], Pierre-Marie Martin[3], Dominique Figarella-Branger[1,5] and L'Houcine Ouafik[1,3]

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[1] *INSERM UMR 911, Faculté de médecine Timone, 13385 Marseille, France*

[2] *Service de Neurochirurgie, département de Neurochirurgie, Hôpital de la Timone ; 13385 Marseille, France*

[3] *Laboratoire de Transfert d'Oncologie Biologique, Faculté de Médecine Nord, 13015 Marseille, France*

[4] *Unité d'Aide Méthodologique à la Recherche Clinique et Epidémiologique, Faculté de médecine Timone, 13385 Marseille, France*

[5] *Service d'Anatomie Pathologique et de Neuropathologie, Hôpital de la Timone, 13385 Marseille, France*

** These authors contributed equally to this work*

Corresponding author: Doctor Philippe Metellus

Service de Neurochirurgie de l'Hôpital de la Timone

264 rue Saint-Pierre, 13385 Marseille cedex 05 France

Phone : + 33 4 91 38 55 45, Fax : + 33 4 91 49 25 18

e-mail: philippe.metellus@mail.ap-hm.fr

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Abstract

Clinical and experimental studies suggest that angiogenesis is a prerequisite for solid tumor growth. Glioblastoma (GBM) and pilocytic astrocytoma (PA), both angiogenic tumors display strong contrast enhancement associated with peripheral oedema in GBM but not in PA indicating differences in vascular permeability in these two types of gliomas. Here we show that expression of adrenomedullin (*AM*) mRNA is induced in GBM whereas is barely detectable in PA. *In situ* analysis of tumor specimens undergoing neovascularization show that the production of AM is specifically induced in a subset of GBM cells distinguished by their immediate proximity to necrotic foci (presumably hypoxic regions), suggesting a hypoxic induction of AM expression in GBM. Vascular endothelial growth factor (*VEGF*) mRNA levels are increased in GBM and moderate in PA. Immunohistochemical study showed that cytoplasmic AM, VEGF and HIF-1α nuclear immunoreactivity was recorded in GBM located near large necrotic areas whereas they were not expressed by PA tumor cells. Interestingly, double fluorescence immunostaining demonstrated that 85% of AM immunoreactivity colocalized with VEGF. AM transduces its effects through calcitonin receptor-like receptor / receptor activity modifying protein-2 and -3 (CLR/RAMP2 and CLR/RAMP3). Real-time quantitative RT-PCR showed expression of *RAMP2*, *RAMP3* and *CLR* in PA and GBM, suggesting that AM may function as an autocrine/paracrine growth factor for GBM cells. These observations strongly support the concept that tumor angiogenesis is regulated by paracrine mechanisms and identify beside VEGF, AM as a potential tumor angiogenesis factor *in vivo* which constitutes a potential interesting molecular target in GBM treatment.

Key words: adrenomedullin (AM); angiogenesis; calcitonin receptor-like receptor (CLR), hypoxia-inducible factor 1 (HIF-1α□); receptor activity modifying-protein (RAMP); vascular endothelial growth factor (VEGF)

Introduction

Glioblastoma multiforme (GBM, WHO grade IV), the most frequent and highly malignant primary brain tumor preferentially affects adults and due to its infiltrative behaviour it cannot be completely resected (1). In contrast, pilocytic astrocytoma (PA, WHO grade I), the most frequent glioma in children is circumscribed and curred by total surgical excision. It occurs mainly in the cerebellum, hypothalamo-chiasmatic region and brainstem (2). Despite highly distinctive features, both tumors are angiogenic and display strong contrast enhancement associated with peripheral oedema in GBM but not in PA indicating differences in vascular permeability in these two types of gliomas. Microvascular proliferation is a histopathological hallmark of GBM and consists of tufted aggregates of dividing endothelial cells, smooth muscle cells and pericytes (3). However, endothelial coverage by pericytes is incomplete in GBM (4), a feature of microvessel immaturity (5). In addition to microvascular proliferation, foci of necrosis surrounded by poorly differentiated cells in a pseudopalisading pattern are another hallmark of GBM (1). In contrast, microvascular proliferation rarely occurs in PA except at the edges of cyst walls but vessels are abundant and typically display hyalinized walls.

Numerous studies have reported that hypoxia is responsible for both pseudopalisading necrosis and angiogenesis in GBM (6). Hypoxia is responsible for activation of hypoxia-inducible factor 1 (HIF-1), a transcription factor which binds to hypoxia-responsive elements (HRE) of various target genes in reduced oxygen condition (7-9). In GBM, the heterodimer HIF-1α/HIF-1β is responsible for the up-regulation of vascular endothelial growth factor (VEGF) and it has been reported to up-regulate adrenomedullin (AM) gene expression under hypoxia in T98G cells (10). Hypoxic induction of VEGF is considered to be the major driving force behind new vessel

development both during embryogenesis and tumor angiogenesis and the VEGF signalling pathway is the major target of currently available anti-angiogenic therapies (11).

AM is a multifunctional peptide with properties ranging from inducing vasorelaxation to acting as a regulator of cellular growth (12). AM transduces its effects through the G protein-coupled receptor calcitonin receptor-like receptor (CLR), with specifity for AM being conferred by the receptor activity modifying protein-2 (RAMP2) and -3 (RAMP3) (13). The ability of CLR/RAMP2 and CLR/RAMP3 to respond with high affinity to AM implies the existence of two molecularly distinct AM receptors referred to as AM_1 and AM_2 receptors, respectively (14). AM is expressed in a variety of malignant tissues and was shown to be mitogenic for human cancer cell lines including lung, breast, colon, glioblastoma and prostate lineages *in vitro* (15, 16).

The aim of this study was to analyze the expression of AM and its receptors CLR, RAMP2 and RAMP3 in a large cohort of GB and PA in order to evaluate the molecular differences between the vasculature of these two angiogenic gliomas. In addition, we analyzed AM topographic distribution using *in situ* hybridization and immunohistochemistry in comparison to those of VEGF and HIF-1α. Finally, we assessed in two glioblastoma cell lines (U87, U373) and in primary glial cell cultures, the effects of hypoxia on AM expression.

Materials and Methods

Human tumor samples

All patients were operated at the same institution (Assistance Publique-Hôpitaux de Marseille, Marseille, France) and written informed consent was obtained in each case. All tissue procurement protocols were approved by the relevant institutional committees. Molecular analysis using real-time quantitative-RT-PCR (Q-RT-PCR) was

performed on 69 frozen gliomas classified according to the WHO CNS tumor classification (1) as glioblastomas (GBM, grade IV, n = 52) and pilocytic astrocytomas (PA, grade I, n = 17). In addition, five GBM samples and three PA samples were fixed in 4% paraformaldehyde, cryoprotected in 20% sucrose, then frozen in melting isopentane and stored at -80°C for *in situ* hybridization analysis. The same samples were formalin-fixed and paraffin-embedded for immunohistochemistry and immunofluoresence studies.

RNA preparation and real-time quantitative RT-PCR (Q-RT-PCR)

Histological control on cryostat section was always realized before tumor pulverization. All PA contain 100% of tumoral tissue whereas GBM contain at least 60% of tumoral tissue and less than 40% necrosis. Total RNA was extracted with a phenol-chloroform method (17) as previously described (18). Forward and reverse primers and probes to quantify genes with their corresponding PCR conditions are described in Table 1.

***AM in Situ* Hybridization**

In situ hybridization using 35S-labeled riboprobes was performed as described previously (19). Observation was done in a Leitz DMRD (Wetzlar, Germany) light microscope equipped with a planachromatic 20x/0.50 objective. Representative microscopic fields were captured as indicated for immunocytochemistry, except that each field was captured twice: once under bright field microscopy to assess histopathological characteristics and once under dark-field microscopy for detecting the distribution of the radioautography silver grains.

Immunohistochemistry of HIF-1*α*, AM and VEGF

We examined the expression of HIF-1α, AM and VEGF proteins in GBM patients (n = 5) who present high expression of AM and VEGF by Q-RT-PCR analysis. PA were taken as negative control (n = 3). Immunohistochemistry was carried out on formalin-fixed paraffin-embedded samples. Five µm sections were tested after heat-induced antigen retrieval (97°C, 40 min, citrate pH 6 buffer). Anti-AM antibody was used at 1/750 dilution (16) and incubated overnight at 4°C before using an avidin-biotin complex kit (Histostain plus, Zymed). Endogen peroxidase was neutralized by 3% H_2O_2. Anti-HIF-1α polyclonal antibody (1/500, gift from J. Pouyssegur, Nice, France; (20)) and anti-VEGF antibody (1/100; RD systems, France) were used for immunostaining performed using a Ventana Automate (Ventana Medical Systems SA, Illkirch, France).

Double immunofluorescence staining of AM and VEGF in GBM

Ten µm sections of GBM samples were incubated in 5% albumin from bovine serum / PBS (Sigma-Aldrich) for 30 min. They were then incubated with the primary antibody mixture (AM diluted at 1/1000; VEGF diluted at 1/100) in 2% bovine serum albumin and 0.1% Triton X-100 in PBS for 1h at room temperature and the secondary antibody anti-goat IgG FITC conjugate and the anti-rabbit Texas Red conjugate (Jackson) diluted at 1/100. Sections were visualized under a Leica (DLMB) immunofluorescence microscope. The staining was semi-quantitatively assessed to determine the percentage of labelled cells.

Western blot analysis

Aliquots of protein extracts (40 µg) of GBM tissues (n = 2) were separated on 12% of SDS-PAGE, transferred to Hybond-C membrane and immunoblotted using antibodies generated against CLR, RAMP2 and RAMP3 peptides as previously described (21).

Cell culture and hypoxia study.

Human glioblastoma cell lines were obtained from the American Type Culture Collection (Rockville, MD) and maintained in minimum essential medium (U373 and U87). Cells were cultured at 37°C under a moist 5% CO_2/95% air atmosphere, and fed with fresh medium every 2 days, being routinely monitored for mycoplasma contamination (Roche Molecular Biochemicals, Meylan, France). All experiments were begun at about 70% confluency. To examine the effect of hypoxia on the levels of AM mRNA and immunoreactive AM (ir-AM), U373 and U87 cells were placed in a chamber filled with 5% CO_2/94% N2/1% O_2 at 37°C. The cells were cultivated for 6 to 24 h and were harvested for RNA extraction. The culture medium was also collected for the measurement of ir-AM. In another series of experiments, cells were exposed to 150 µmol/L of cobalt chloride ($CoCl_2$) or 260 µmol/L of desferrioxamine mesylate (DFX) for 6 to 24 h and then harvested for RNA extraction. All culture media components were purchased from Invitrogen Life Technologies (Paris, France).

Peptide Extraction and Radioimmunoassay.

Medium from a primary tumoral glial cells and U87 cell line were prepared for radioimmunoassay (RIA) of AM as previously described (16). The RIA of AM was performed as reported previously (22), using the antiserum against human (AM 1–52) amide developed in our laboratory and used at a final dilution of 1:30,000. To measure

the ir-AM in the culture medium, the medium was extracted by the previously reported method (22) using Sep-PaK C18 cartridges (Waters, Milford, MA).

Statistical analysis

Statistical analyses were conducted using the statistical Package SPSS software v.15 (SPSS Inc, Chicago, USA). Quantitative RT-PCR data were expressed as mean ± the standard error of the mean (SEM), and differences between GBM and normal telencephalon were analyzed using the Mann–Whitney U test. The difference was considered significant if the *p*-values were less than 0.05.

Results

Increased expression of *AM* and *VEGF* mRNAs in glioblastomas (GBM) in comparison to pilocytic astrocytomas (PA).

Quantification of *AM* mRNA transcripts on large number of samples revealed high *AM* mRNA levels in GBM as compared to PA, in agreement with our previous data on small number of GBM (16). The *AM* mRNA expression analysis demonstrated a significant difference between GBM and PA (58.2 ± 8 fg/ng 18S rRNA in GBM versus 5.9 ± 1.7 fg/ng 18S rRNA in PA ($p < 0.0001$)) (Figure 1A). The mean level of *VEGF* mRNA was increased in GBM (162.3 ± 19 fg/ng 18S rRNA) as compared to PA (19.9 ± 5.7 fg/ng 18S rRNA, $p < 0.0001$) (Figure 1A). *VEGF* mRNA expression was correlated with *AM* mRNA expression in PA and GBM (respectively, 0.75, $p < 0.001$; 0.56, $p < 0.0001$, Pearson Correlation Test).

To determine whether AM was predominantly expressed in a restricted subpopulation of tumor cells, *in situ* analysis of *AM* mRNA was performed to identify the producer cells. In figure 1B, *AM* mRNA is seen to be mainly produced in a fraction of tumor cells

(presumably experiencing the most severe hypoxia) arranged in a stripe-like pattern alongside the periphery of necrotic regions. The same finding has been previously reported for VEGF expression in GBM (23).

Distribution of HIF-1α, AM and VEGF immunostaining in GBM.

HIF-1α, AM and VEGF proteins expressions were recorded in tumor cells in all samples studied although the proportion of cells expressing HIF1-α, AM and VEGF varied considerably between tumors. As expected, HIF1-α nuclear immunoreactivity was expressed frequently in GBM cells located near large necrotic areas and in some pseudopalisading tumor cells (Figure 2A a and d) as well as cytoplasmic AM (Figure 2A b and e) and VEGF (Figure 2A c and f). Interestingly, double fluorescence immunostaining demonstrated clearly that in GBM, 85% of AM immunoreactivity colocalized with VEGF (Figure 2B) whereas 6% and 9% of cells exclusively expressed AM or VEGF, respectively. Moreover, immunostaining of HIF-1α, AM and VEGF were not recorded in PA tumor cells (data not shown).

RAMPs and CLR are expressed in GBM and PA.

Figure 3A shows that *CLR, RAMP2* and *RAMP3* mRNAs were expressed in all of the samples prepared from GBM and PA. No significant difference in the expression of *CLR, RAMP2* and *RAMP3* mRNAs could be observed between GBM and PA. Omission of the reverse transcriptase eliminated the signal, which indicated that it was not attributable to contaminating genomic DNA (data not shown).

To confirm expression of CLR, RAMP2 and RAMP3 proteins, protein extracts (40 µg) from tumor cells of two GBM samples were subjected to western blot analysis (Figure

3B). The specificity of our immunodetection assay was confirmed by an antibody adsorption control that eliminated the specific bands (data not shown).

Taken together, these findings demonstrate that CLR, RAMP2, and RAMP3 mRNAs and proteins are expressed in GBM tumoral cells and may contribute to the function of the AM.

Regulation of AM expression in human glioblastoma cells by hypoxia.

The two cell lines (U373, U87) exposed to hypoxia, $CoCl_2$, and DFX demonstrated a consistent induction of AM expression, and fig. 4 illustrates a representative example of the observed response in our tests conditions (hypoxia, exposure to 150 µmol/L $CoCl_2$ and to 260 µmol/L DFX).

Of the three treatments, exposure to 1% O_2 and to DFX showed a steeper induction of AM mRNA over time, and also more dramatic increases between the basal and the maximum induction was observed (>28- and 25-fold increase between maximum induction and baseline levels for exposure to 1% O_2 and DFX, respectively).

We also determined the ir-AM in the conditioned medium of U373 and U87 cells and primary glial cell culture prepared from 8 GBM specimens under hypoxic condition. ir-AM accumulated in the culture media of U373 and U87 cells and primary glial cell culture time-dependently up to 24h under normoxia and hypoxia. The ir-AM levels were significantly higher under hypoxia than under normoxia in primary glial cell culture (fig. 5), U87 (151.6 +/- 22.4 *v* 47.9 +/- 12.1 fmol/10^6 cells) and U373 (169.5 +/- 27.3 *v* 54.7 +/- 9.6 fmol/10^6 cells) cells (not shown). Similarly results were obtained with the conditioned media of these cell lines for $CoCl_2$ or DFX treatments (not shown).

Discussion

In this paper, we reported a strong AM expression in GBM comparing to PA ($p < 0.001$). In contrast, RAMP2 and RAMP3 expression were in the same range in both tumors whereas CLR expression was higher in PA than in GBM ($p < 0.01$). High AM expression in GBM in contrast to grade II and III infiltrative glioma was previously reported by our group, but at this time we did not study AM expression in PA (16). The marked differences in steady-state levels of *AM* mRNA among otherwise indistinguishable tumor cells (Figure 1B) can be correlated with their proximity to necrotic centres, where oxygen supply is minimal. This observation can be interpreted to mean that AM could be specifically induced in response to hypoxia. Interestingly, Garayoa and coworkers (24) demonstrated that the expression of *AM* mRNA in a variety of human cell lines is highly induced by hypoxia. Here, we also demonstrated that reduced oxygen tension (1% O_2) or exposure to hypoxia mimetics such as desferrioxamine mesylate (DFX) or $CoCl_2$, induced *AM* mRNA expression in U87 and U373 GBM cell lines. The increase in *AM* mRNA levels was reversible. Upon re-exposure of cells to normal oxygen tension, AM expression resumed its low constitutive level (not shown). These findings support the thesis that the induction of AM in GBM occurs in response to hypoxia. They also indicate that the rate of release of angiogenic factors (AM, VEGF...) by tumor cells might in general be variable, being constantly adjusted according to the changes in the cell microenvironment.

Evidence for both AM and AM receptors in GBM favour that AM functions as autocrine/paracrine growth factor in GBM. In agreement, anti-AM antibody significantly decreased *in vitro* and *in vivo* growth of U87 glioblastoma cells (16). In addition, to its role in tumor progression, AM knockout mice suggest that AM is essential for vascular morphogenesis (25) and also tumour angiogenesis (26). In various tumour models, AM expression correlates with vascular density (16, 27) but highly

vascularized tumor may express low level of AM as reported here for PA. In fact, the mechanism leading to angiogenesis in both GBM and PA are strikingly different: in PA, which is a slowly growing tumor, preexisting blood vessels are sufficient to ensure tumor growth. Vessels diameter increased and become abnormal with hyalinized walls as tumors enlarged. In contrast, in rapidly growing GBM, blood supply is not sufficient leading to necrosis and hypoxia which is the major trigger of angiogenesis. Increased AM expression in hypoxic conditions occurs through increase transcription factor HIF-1 binding to HRE in the promoter of the AM gene but also through increase of AM mRNA stability that takes place during hypoxia (28). Under hypoxic conditions, VEGF is also upregulated through HIF-1 binding (9). In addition, AM administration upregulates the expression of VEGF in both *in vivo* and *in vitro* models (26). In contrast, blocking antibodies to VEGF cannot significantly inhibit AM induced capillary tube formation by HUVEC indicating that AM expression is to some extent independent to VEGF (29). In our study AM and VEGF mRNA expression was highly correlated ($p<0.001$) but strikingly different from one GBM to another. Moreover, immunohistochemistry shows that the expression pattern of these markers varies from one GBM to another. In some cases it was diffuse but in others, restricted to some areas exhibiting palisading necrosis. In these areas, the pattern of VEGF and AM expression observed in GBM only partly overlap with that of HIF-1α; HIF-1α expression was restricted to some pseudopalisading tumor cells adjacent to necrosis whereas AM and especially VEGF expression was more widespread.

Tumor vessels in GBM are structurally and functionally abnormal. They are tortuous with leaking architecture and irregular diameter and walls and contribute to the pathogenesis of tumor-associated edema. A relative deficiency of pericytes or pericytes function could be responsible for this morphological feature (4). The number of α-

smooth muscle actin expressing cells, a marker of pericytes, was much lower in anaplastic astrocytomas than in PA which is made of large, regularly shaped and still matures vessels (30). However, there are increasing evidences that remaining functional vessels in GBM are at least in part responsible for resistance to anti-angiogenic therapy since inhibition of VEGF signalling can lead to substantial reduction in tumor vascularity but does not affect vessels highly covered with pericytes (31). Therefore, there is a rationale for targeting both endothelial cells and pericytes (32). In contrast to VEGF induced angiogenesis which is not associated with vessel maturation, AM induced angiogenesis facilitates formation of mature vessels that include vascular smooth muscle cells (33). Therefore, there is a rationale for angiogenesis inhibitors targeting the AM signalling pathway in GBM. Moreover, it is possible that AM in GBM contributes to tumor associated edema because of its strong vasodilator action (15). In addition to AM, it has been shown that hypoxia also upregulated CLR expression in microvascular endothelial cells although RAMP expression is not affected by hypoxia in microvascular cells (34). In agreement, RAMP2 and RAMP3 expression did not differ in PA and GBM. Surprisingly however, CLR expression was higher in PA in comparison to GBM suggesting that another factor different from hypoxia may govern CLR expression in PA.

As a conclusion, in PA which is a circumscribed and slow growing tumor, preexisting vessels adapt their caliber and vascular pressure to ensure sufficient blood supply whereas in highly infiltrative and rapidly growing GBM, hypoxia-dependent angiogenesis occurred involving numerous growth factors including AM and its receptors. Targeting AM signalling might be instrumental for anti-angiogenic therapy in these tumors.

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Table and figure legends

Table 1: Sequences of forward and reverse primers and probes used to quantify human *AM*, *VEGF*, *CLR*, *RAMP2*, *RAMP3* mRNAs, and *18S* rRNAs. Corresponding Q-RT-PCR conditions are also given.

Figure 1: Expression of AM and VEGF in GBM and PA. (A) Real time quantitative RT-PCR analysis of *AM* and *VEGF* mRNA levels in GBM and PA. Total DNA-free RNA from the GBM (n = 52) and PA (n = 17) were transcribed to cDNA and subjected to quantitative RT-PCR using the ABI Prism 7700 sequence detection system for the estimation of relative *AM* and *VEGF* mRNA to 18S rRNA ratio as described in Materials and Methods. **(B)** Radioactive *in situ* hybridization of AM mRNA in a human GBM. A thin section of GBM tumor hybridized with the sense AM riboprobe showing no signal (high specificity) at magnification x 20 **(a)** et x 40 **(b)** and with the antisense AM riboprobe at magnification x 20 **(c)** showing a strong signal. AM expressing cells are localized alongside the edges of necrotic regions (N) and a high endothelial proliferation can be observed at the periphery of these zones **(d)**. Furthermore, the clusters of silver grains concentrate within tumor cells rather than in endothelial cells **(d)**.

Figure 2: HIF-1α, AM and VEGF proteins are present in GBM in vivo. (A) Immunocytochemical staining for HIF-1α **(a)**, AM **(b)** and VEGF **(c)** shows strong immunoreactivity in palissading cells that reside along necrosis. **(d-f)** Enlargement of the areas boxed in a, b and c, respectively. **(B)** Immunofluorescence of AM and VEGF demonstrates that in GBM, 85% of AM immunoreactivity colocalize with VEGF.

Figure 3: Expression of CLR, RAMP2 and RAMP3 in GBM and PA. (A) Real-time quantitative RT-PCR analysis of CLR, RAMP2 and RAMP3 mRNA levels an GBM (n = 52) and PA (n = 17). Total RNA DNA-free from GBM and PA was reverse transcribed and quantified as described in figure 1 for the estimation of relative *CLR*, *RAMP2* and *RAMP3* mRNAs to 18S rRNA ratio as described in Materials and Methods. In GBM, the mean level of CLR, RAMP2 and RAMP3 mRNAs expression was 9.9 ± 0.9 fg/ng 18S rRNA, 9.3 ± 0.9 fg/ng 18S rRNA, and 5.6 ± 1.1 fg/ng 18S rRNA, respectively. In PA, the mean level of *CLR*, *RAMP2* and *RAMP3* mRNAs expression was 13.9 ± 1.6 fg/ng 18S rRNA, 7.7 ± 1.1 fg/ng 18S rRNA, and 6.2 ± 1.3 fg/ng 18S rRNA respectively. No significant difference in the expression of *CLR*, *RAMP2* and *RAMP3* mRNAs could be observed between GBM and PA. **(B)** Aliquots of protein extracts (40 µg) of GBM tissues (n = 2) were separated on 12% of SDS-PAGE, transferred to Hybond-C membrane and immunoblotted using antibodies generated against CLR, RAMP2 and RAMP3 peptides as described (21). GBM produced CLR as a distinct band of 48 kda after SDS-PAGE and immunoblotting (Figure 3B, lanes 1 and 2). RAMP2 ran as a monomer of 15 kda and multimer presumably homodimer at 50 kda (Figure 3B, lanes 3 and 4). The 35 kda band may represent a heterogeneously glycosylated form of RAMP2 (Figure 3B, lanes 3 and 4). RAMP3 ran as an heterogeneously glycosylated monomer of 28 kda and an heterodimer of 73-Kda (Figure 3B, lanes 5 and 6). Interestingly, the 73 kda band can be revealed by the three antibodies and must correspond to heterodimers CLR/RAMP2 and CLR/RAMP3 (Figure 3B).

Figure 4 (A): Hypoxic induction of AM mRNA in U87 (A) and U373 (B) glioblastoma cells. The intensity of hybridization signals of northern blot analysis was

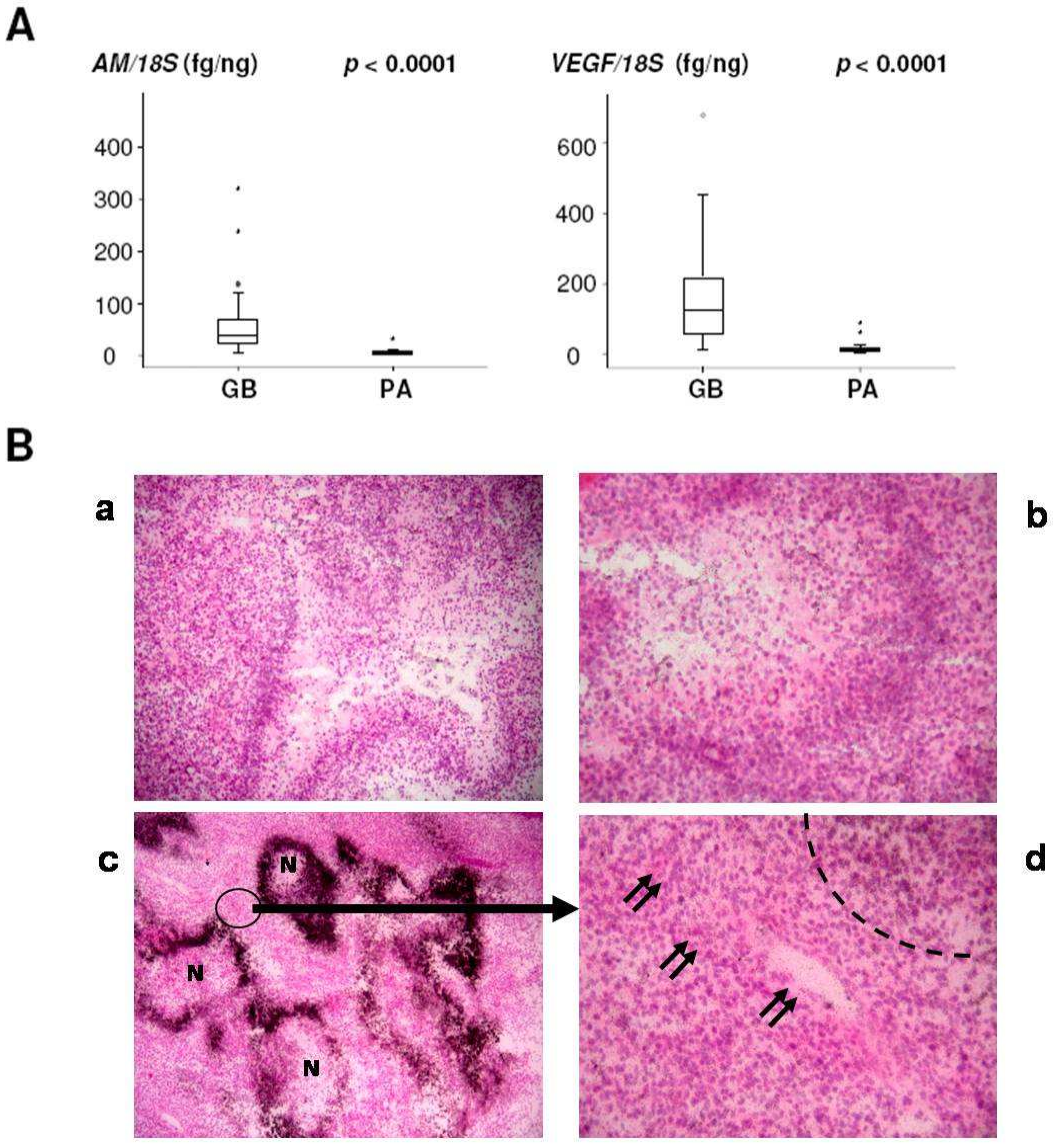
quantified with a Bioimage Analyzer, and the intensity of representing AM mRNA was normalized with respect to the intensity for 18S r-RNA. The ratio of each normalized value to the control value (before hypoxic experiments) is shown as the relative expression level of AM mRNA. The data are means +/- SEM. The time course study revealed that expression levels of *AM* mRNA increased significantly after 6-h exposure to hypoxia and reached the maximum at the 24-h time point (about 32- and 28-fold increase compared to control for U87 and U373 respectively). In the hypoxia mimetics experiment ($CoCl_2$, DFX), the difference of AM mRNA expression was significant at 16-h time point and reached the maximum at the 24 h time point. The mean calculated test/basal ratio was of 14- and 9-fold in $CoCl_2$ exposure experiments for U87 and U373 cell lines respectively. It was of 37- and 25-fold DFX exposure experiments for U87 and U373 cell lines respectively.

Figure 5: Effect of hypoxia on AM protein level in primary glial cell culture prepared from 8 human glioblastoma specimens. IR-AM levels in the culture medium under normoxic and hypoxic conditions. The data shown are means +/- SEM. $P<0.005$

Gene	Primers, fluorescent probes, PCR conditions
AM	Forward primer 5'-TGCCCAGACCCTTATTCGG-3', Reverse primer 5'-AGTTGTTCATGCTCTGGCGG-3' Probe FAM-ACATGAAGGGTGCCTCTCGAAGCCC-TAMRA (95℃ for 15'; 45 cycles of 94℃ for 15", 67℃ for 20")
VEGF	Forward primer 5'-AGGAGGAGGGCAGAATCATCA-3' Reverse primer 5'-AGGGTCTCGATTGGATGGC-3' Probe FAM-TGAAGTTCATGGATGTCTATCAGCGCAGCT-TAMRA (95℃ for 15'; 45 cycles of 94℃ for 15", 66℃ for 15")
CLR	Forward primer 5'-TGGCTTAATGATGGAGAAAAAGTG-3' Reverse primer 5'-TCAGGACTCTCTTCTAATTCTGCTG-3' Probe FAM-CCTGTATTTTCTGGTTCTCTTGCCTTTTTTTTATGA-TAMRA (95℃ for 15'; 40 cycles of 94℃ for 20", 60℃ for 20")
RAMP2	Forward primer 5'-GACGGTGAAGAACTATGAGACAGC-3' Reverse primer 5'-GCTATAAGGCCTGCTAATCATGG-3' Probe FAM-TGGATCCTATCGAAAAGGATTGGTGCG-TAMRA (95℃ for 15'; 40 cycles of 94℃ for 10", 65℃ for 15")
RAMP3	Forward primer 5'-TCTGGAAGTGGTGCAACCTGT-3' Reverse primer 5'-GATGCCGGTGATGAAGCC-3' Probe FAM-AGATGGAGGCCAATGTCGTGGGCT-TAMRA (95℃ for 15'; 40 cycles of 94℃ for 20", 67℃ for 30")
18S	Forward primer 5'-CTACCACATCCAAGGAAGGCA-3' Reverse primer 5'-TTTTTCGTCACTACCTCCCCG-3' Probe FAM-CGCGCAAATTACCCACTCCCGAC-TAMRA (95℃ for 15'; 40 cycles of 94℃ for 15", 67℃ for 15")

FAM, 5-carboxyfluorescein; TAMRA, 5-carboxytetramethylrhodamine

Table 1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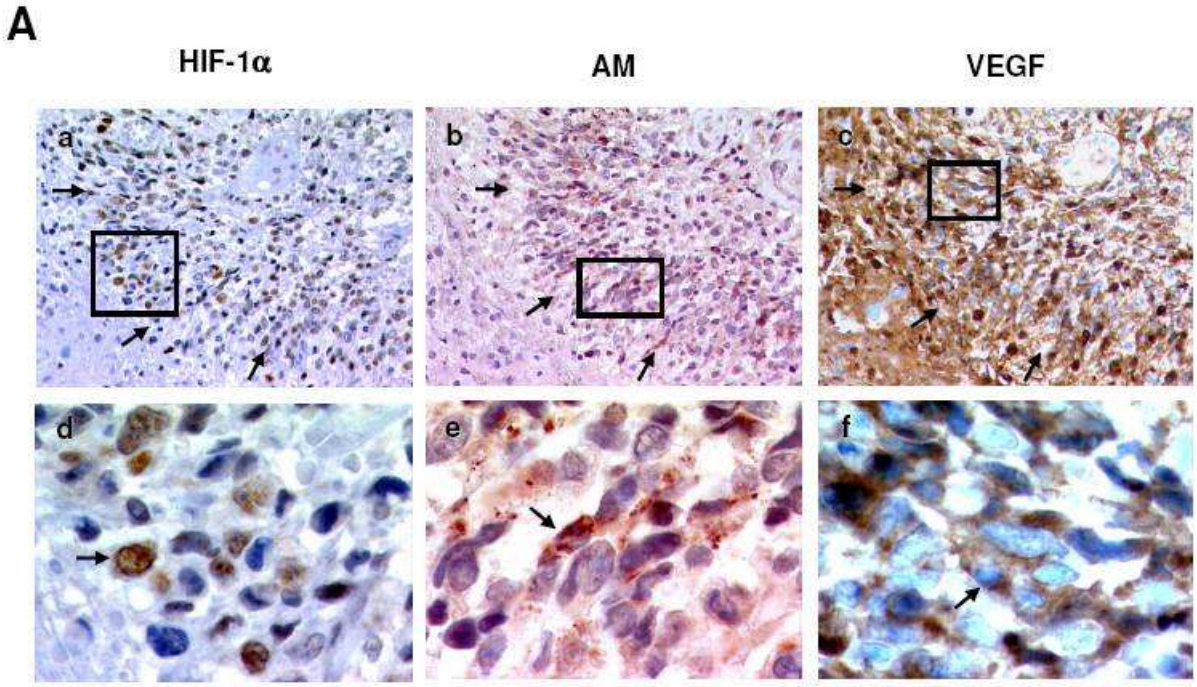
AM/18S (fg/ng)
p < 0.0001
400
300
200
100
0
GB
PA
VEGF/18S (fg/ng)
p < 0.0001
600
400
200
0
GB
PA
B
a
b
c
d
N
N
N

Figure 1



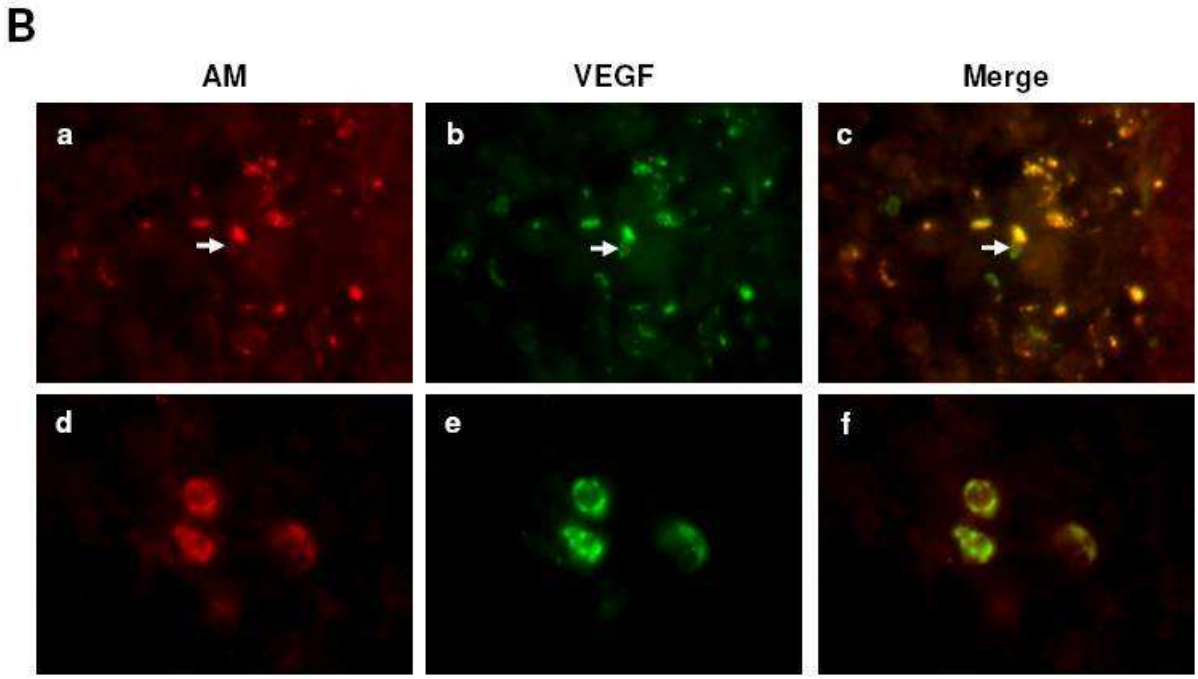


Figure 2

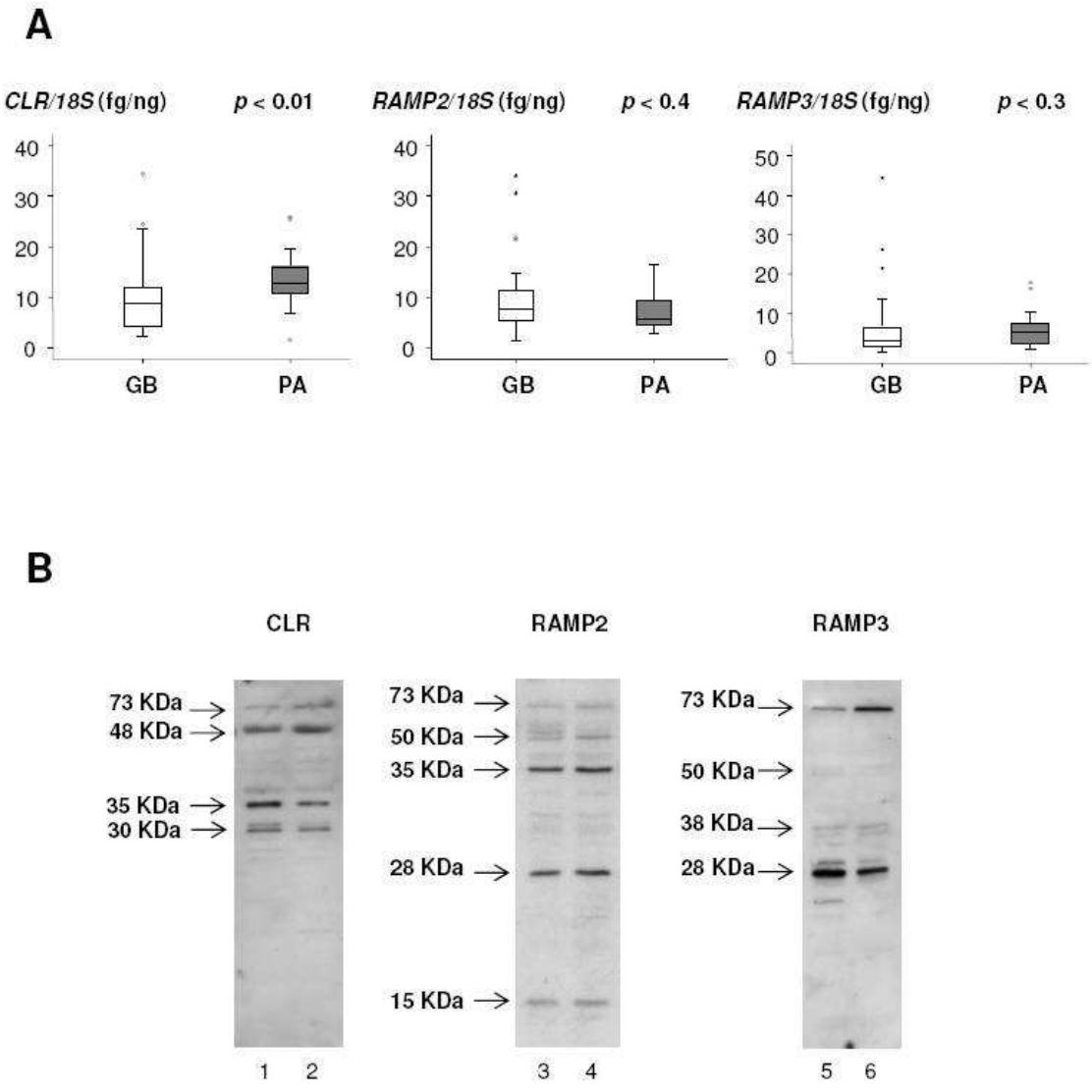
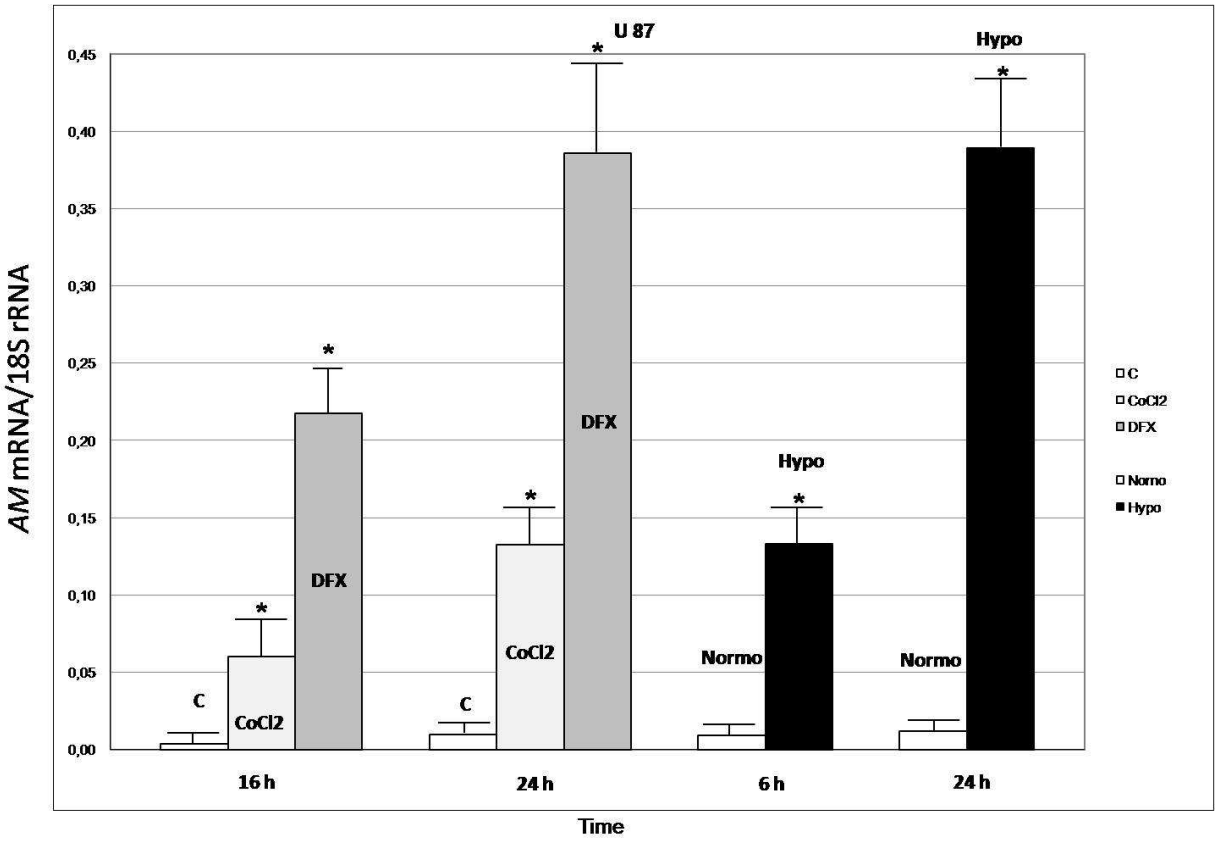

A
CLR/18S (fg/ng)
p < 0.01
RAMP2/18S (fg/ng)
p < 0.4
RAMP3/18S (fg/ng)
p < 0.3
40
30
20
10
0
50
GB
PA
B
CLR
RAMP2
RAMP3
73 KDa
48 KDa
35 KDa
30 KDa
50 KDa
28 KDa
15 KDa
38 KDa
1 2 3 4 5 6

Figure 3


U 87
Hypo
AM mRNA/18S rRNA
0,45
0,40
0,35
0,30
0,25
0,20
0,15
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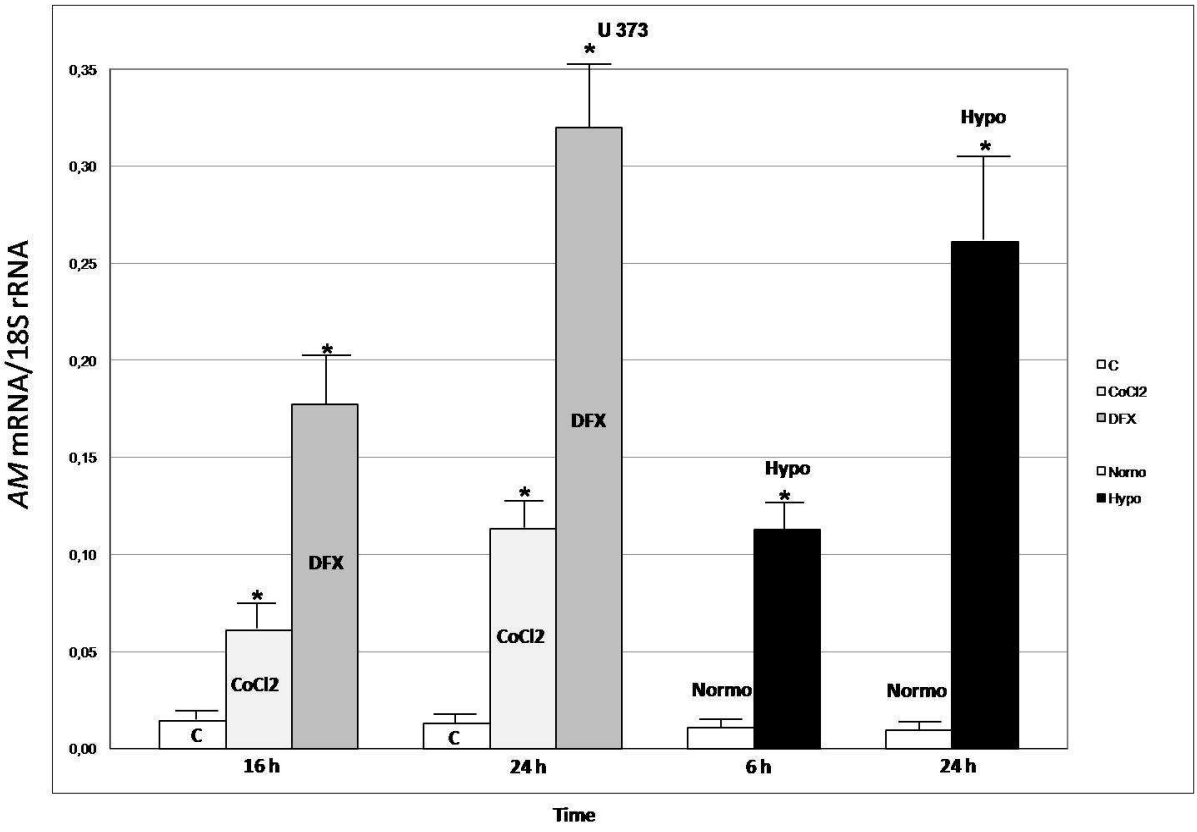
0,00
C
CoCl2
DFX
C
CoCl2
DFX
Hypo
Normo
Normo
Hypo
16 h
24 h
6 h
24 h
Time
C
CoCl2
DFX
Norno
Hypo

Figure 4A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AM mRNA/18S rRNA
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0,25
0,20
0,15
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C
CoCl2
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Hypo
16 h
24 h
6 h
24 h
Time

Figure 4B

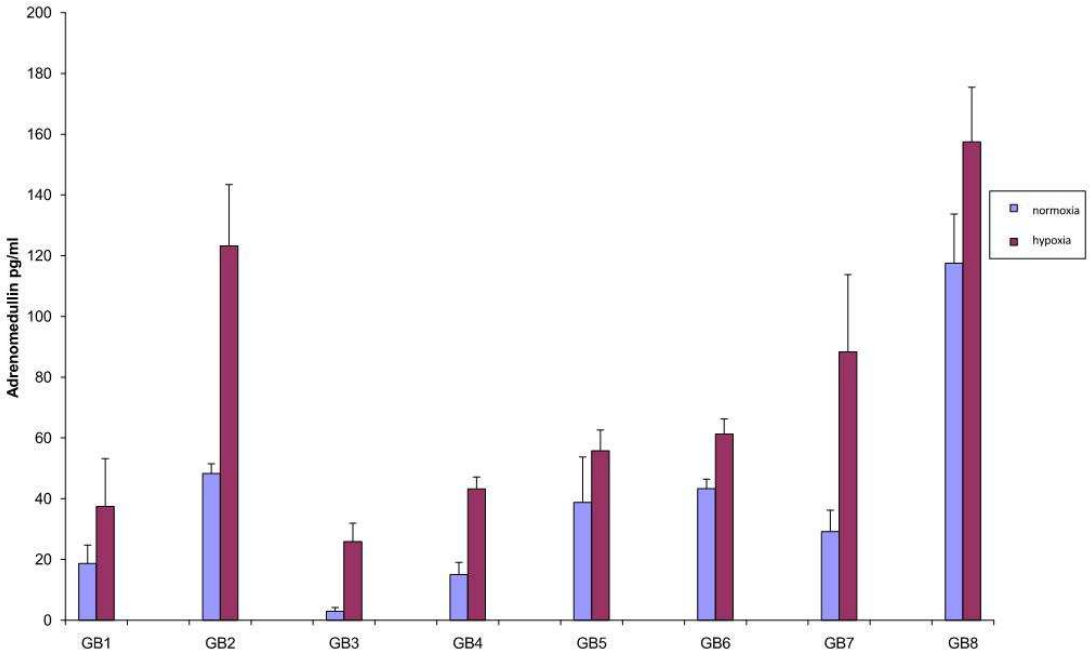
Adrenomedullin pg/ml
200
180
160
140
120
100
80
60
40
20
0
GB1
GB2
GB3
GB4
GB5
GB6
GB7
GB8
normoxia
hypoxia

Figure 5

PUBLICATION 2

Targeting adrenomedullin receptors with systemic delivery of neutralizing antibodies inhibits tumor angiogenesis and suppresses growth of human tumor xenografts in mice.

Kaafarani I, Fernandez-Sauze S, Berenguer C, Chinot O, Delfino C, Dussert C, Metellus P, Boudouresque F, Mabrouk K, Grisoli F, Figarella-Branger D, Martin PM, Ouafik L.

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Targeting adrenomedullin receptors with systemic delivery of neutralizing antibodies inhibits tumor angiogenesis and suppresses growth of human tumor xenografts in mice

Itûdal Kaafarani,*,† Samantha Fernandez-Sauze,*,† Caroline Berenguer,*,† Olivier Chinot,*,† Christine Delfino,*,† Christophe Dussert,*,† Philippe Metellus,*,† Françoise Boudouresque,*,† Kamel Mabrouk,§ François Grisoli,‖ Dominique Figarella-Branger,* Pierre-Marie Martin,*,†,‡ and L'Houcine Ouafik*,†,‡,1

*INSERM, Unité Mixte de Recherche (UMR) 911-CRO2, Marseille, France; †Aix Marseille Université, Faculté de Médecine Secteur Nord, Laboratoire de Cancérologie Expérimentale, Marseille, France; ‡Laboratoire de Transfert d'Oncologie Biologique [Assistance Publique—Hôpitaux de Marseille (AP-HM)], Marseille, France; §Centre National de la Recherche Scientifique, UMR 6264, Aix Marseille Université, Faculté de Saint Jérôme, Equipe Chimie Radicalaire Organique et Polymère de Specialité (CROPS) Laboratoire de Chimie Provence, Marseille, France; and ‖Service de Neurochirurgie (AP-HM), Hôpital Timone, Marseille, France

ABSTRACT Adrenomedullin (AM) is a multifunctional peptide vasodilator that transduces its effects through calcitonin receptor-like receptor/receptor activity modifying protein-2 and -3 (CLR/RAMP2 and CLR/RAMP3). Previously, we reported on the development of an anti-AM antibody that potently inhibits tumor cell proliferation *in vitro* and tumor growth *in vivo*. Here, we report the effect of anti-AM receptor antibodies (αAMRs) on angiogenesis and tumor growth. We demonstrate that αAMRs decrease in a dose-dependent manner the growth of U87 glioblastoma cells and HT-29 colorectal cancer cells, but not A549 lung cancer cells, *in vitro*. *In vivo*, AM in Matrigel plugs induces angiogenesis by promoting recruitment of endothelial cells, pericytes, myeloid precursor cells, and macrophages and by promoting channel formation. Remarkably, systemic administration of αAMRs every 3 d markedly reduced neovascularization of Matrigel plugs in a dose-dependent fashion, as demonstrated by reduced numbers of the recruited cells and vessel structures. Several human tumor xenografts in athymic mice were used to examine the effect of αAMR treatment on tumor angiogenesis and growth. αAMR treatment significantly suppressed the growth of glioblastoma, lung, and colon tumors. Histological examination of αAMR-treated tumors showed evidence of disruption of tumor vascularity with decreased microvessel density, depletion of endothelial and pericyte cells, and increased tumor cell apoptosis. These findings support the conclusion that αAMR treatment inhibits tumor growth by suppression of angiogenesis and tumor growth and suggest that AMRs may be useful therapeutic targets.—Kaafarani, I., Fernandez-Sauze, S., Berenguer, C., Chinot, O., Delfino, C., Dussert, C., Metellus, P., Boudouresque, F., Mabrouk, K., Grisoli, F., Figarella-Branger, D., Martin, P.-M., Ouafik, L. H. Targeting adrenomedullin receptors with systemic delivery of neutralizing antibodies inhibits tumor angiogenesis and suppresses growth of human tumor xenografts in mice*FASEB J.* 23, 000–000 (2009). www.fasebj.org

Key Words: CLR/RAMP2 · CLR/RAMP3 · endothelial cells · pericytes · proangiogenic cells

TUMORS INFLUENCE THE SURROUNDING host stroma by inducing angiogenesis to supply their oxygen and nutrient needs. The angiogenesis or formation of new blood vessels out of preexisting capillaries is a sequence of events that is of key importance in a broad array of physiological and pathological processes in response to spontaneous or induced tissue hypoxia (1). Tumor angiogenesis is regulated by a balance of pro- and antiangiogenic molecules. When the balance shifts in favor of angiogenesis inducers, an angiogenic switch activates the normally quiescent vasculature to develop new blood vessels (2). During the final maturation stages, the endothelial cells acquire a more differentiated state marked by lumen formation, production, and assembly of a complex basement membrane and junction with pericytes. Finally, periendothelial cells are recruited into the area, thereby providing further support for the new vessel (2). Increasing evidence suggests that the contribution of hematopoietic stem cells and myeloid progenitor cells is critical for new vessel formation in tumors (3). Once they have homed to the tumor, multipotent bone marrow-derived cells have the

[1] Correspondence: INSERM, UMR 911-CRO2, Faculté de Médecine Nord, Bd Pierre Dramard, 13916 Marseille Cedex 20, France. E-mail: lhoucine.ouafik@univmed.fr

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potential to modulate neovascularization and to become part of all three nonmalignant tumor compartments: hematopoietic, endothelial, and mesenchymal (4).

Tumor vessels are structurally and functionally abnormal, with defective endothelium, basement membrane, and pericyte coverage (1, 5). One extreme example is glioblastoma multiforme, an invariably fatal brain tumor characterized by a dilated, tortuous, disorganized, and leaky vasculature (6). Due to their critical role for tumor growth, tumor blood vessels are recognized as potential antitumor drug targets (7), and their abnormalities open up the possibility of targeting tumor vessels separately from the vessels of the surrounding normal tissue (8). Several growth factors have been identified as possible regulators of angiogenesis (9). Among these factors, adrenomedullin (AM) is thought to play an important role in tumor angiogenesis (10–12).

AM is a widely distributed multifunctional peptide with properties ranging from inducing vasorelaxation to acting as a regulator of cellular growth (13, 14). Three AM receptors (AMRs) with different affinities for AM [L1, RDC1, and calcitonin receptor-like receptor (CLR)] have been cloned (15–17). All of them belong to the 7-transmembrane domain G-protein-coupled receptor superfamily. Interestingly, CLR requires the presence of modulating proteins with a single transmembrane domain known as receptor activity modifying proteins (RAMPs) (18). RAMP1 presents CLR at the plasma membrane as a calcitonin gene-related peptide (CGRP) receptor, whereas RAMP2 and RAMP3 present CLR as an AMR (18).

The functions of AM discovered due to many physiological studies are highlighted in knockout studies in which AM-null mice die *in utero* from extreme hydrops fetalis and vascular abnormalities (19, 20). Recently, Fritz-Six *et al.* (21) reported that deletion of *AM*, *Calcrl*, or *RAMP2* results in massive cutaneous edema and midgestation lethality in mice due to defects in lymphatic vessel growth. At the same time, Ichikawa-Shindo *et al.* (22) described generalized edema and midgestation death, as well as occasional hemorrhage, in RAMP2-deficient animals. The authors concluded that AM serves critical roles in blood vessels, including regulation of vascular stability and permeability, and that embryos lacking AM signaling die due to leaky and unstable blood vessels.

AM is highly expressed in a variety of malignant tissues and is mitogenic for human cancer cell lines including lung, breast, colon, glioblastoma, pancreas, and prostate lineages *in vitro* (10, 23–27). Evidence for the importance of AM-induced angiogenesis in tumor growth includes the observation that inhibition of AM action by neutralizing antibodies or AM antagonist hAM_{22-52} blocks the growth of tumor xenografts *in vivo* (10, 28). The density of blood vessels in the anti-AM-antibody-treated tumors was also decreased, supporting the roles for AM in angiogenesis and/or vessel stabilization (10, 12). The AM antagonist hAM_{22-52} was demonstrated to inhibit pancreatic cancer cell growth *in vivo* by suppressing tumor vascular development (28).

The involvement of CLR/RAMP2 and CLR/RAMP3 receptors to mediate the effect of AM in the multistep process of angiogenesis and tumoral cell proliferation suggests that blockade of these receptors would be a useful therapeutic strategy for inhibiting angiogenesis and tumor growth. Previously, we described the development of anti-CLR, anti-RAMP2, and anti-RAMP3 polyclonal antibodies targeting the extracellular domain of each protein. These antibodies are potent antagonists for AM binding, AM-induced endothelial cell migration and invasion *in vitro*, and AM-induced capillary-like structures *in vitro* (11). In this study, we analyzed the effect of AMR blockade by anti-AMR polyclonal antibodies (αAMRs) in *in vivo* models of angiogenesis and on tumor growth in several human xenograft tumor models.

MATERIALS AND METHODS

Cell culture

Human glioblastoma U87, colon HT-29, and lung A549 cell lines were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured in Eagle's MEM (EMEM), L15 medium, and DMEM, respectively (Invitrogen Life Technologies Inc., Paris, France) as described previously (10). Cells were cultured under a moist 5% CO_2/95% air atmosphere. All culture medium components were purchased from Invitrogen Life Technologies.

Animals

Six- to 8-wk-old female C57BL/6 and athymic NMRI (*nu/nu*) mice were purchased from Harlan (Gannat, France). Animals were anesthetized before all procedures and observed until fully recovered. Experimental protocols involving animals were reviewed and approved by the Institutional Animal Care Committee of the School of Medicine and performed in accordance with INSERM and Aix Marseille Université School of Medicine polices regarding the use of laboratory animals.

Characterization of anti-human AMR antibodies

The polyclonal antibodies anti-CLR, anti-RAMP2, and anti-RAMP3 referred to here as αCLR, αRAMP2, and αRAMP3, respectively, were developed and characterized as described previously (11). Purified IgG of αCLR, αRAMP2, and αRAMP3 and preimmune serum (rabbit control IgG) were affinity purified on rProtein A Sepharose fast-flow columns (GE Healthcare, Europe GmbH, Orsay, France) and tested for endotoxin using the Pyrogent Plus Limulus ameboycote lysate kit (Lonza, Verviers, Belgium). All antibody preparations used in animal studies contained <1.25 endotoxin U/ml.

Preparation of membranes

Mouse tissues (brain, kidney, and lung) were homogenized in 15 mM HEPES, pH 8.0; 5 mM EDTA; and 5 mM EGTA containing inhibitors (Completes; Roche, Basel, Switzerland) using a Brinkmann Polytron (Brinkmann Instruments, Westbury, NY, USA) at 4°C. The homogenates were centrifuged for 10 min at 1,000 *g* to sediment nuclei and cell debris. The homogenates were then centrifuged for 60 min at 100,000 *g* at 4°C, and the resulting membrane pellet was resuspended in 50 mM HEPES, pH 8.0; 5 mM EDTA; and 5 mM $MgCl_2$ buffer. Protein concentration was determined using the Micro bicinchoninic acid protein assay reagent (Pierce, Interchim, Paris, France) with BSA as the standard.

Western blot analysis

Membrane extracts (40 μg) from mouse tissues were resolved by 10% SDS-PAGE, transferred to hybond-C membranes (GE Healthcare), and immunoblotted with antibodies directed against CLR, RAMP2, and RAMP3 (11). Membranes were then washed with phosphate-buffered saline plus 5% milk with 0.04% Tween 20 (PBS-T) and incubated with 1:30,000 dilution of goat anti-rabbit secondary antibody conjugated to horseradish (GE Healthcare) for 45 min. The membranes were washed with PBS-T, incubated in Chemiluminescent Substrate (GE Healthcare) for 5 min, and exposed to film. Molecular weights were estimated by comparison with standard proteins (Rainbow markers; Invitrogen Life Technologies). Specificity control consisted of a duplicate membrane incubated in antigen-preadsorbed (20 μg/ml) antiserum.

Binding of labeled ^{125}I-AM

Glioblastoma cells were cultured in 24-well plates for 48 h (15×10^4 cells/well) and then serum starved for 24 h. After being washed with PBS, the cells were incubated in TIS medium (MEM plus 10 μg/ml transferrin, 10 μg/ml insulin, and 3×10^{-8} M sodium selenite) at 25°C for 120 min with the radioactive tracer in the presence or absence of an excess (10^{-6} M) of unlabeled AM as described previously (29). Monoiodinated ^{125}I-hAM (specific activity, 1172 Ci/mmol) was purchased from Bachem (Peninsula Labs, Belmont, CA, USA). In binding inhibition studies, cells were incubated with tracer and increasing concentrations of the αAMR antibodies. At the end of the incubation period, cells were extensively washed with cold PBS containing 0.2% BSA, solubilized with 0.2 M sodium hydroxide, and analyzed for bound radioactivity in a γ-spectrometer. Specific binding was obtained by subtracting nonspecific binding in the presence of excess unlabeled hAM from total binding. Data points represent the means of 3 experiments, each performed in triplicate.

Cell proliferation assay

The assay was performed in EMEM with 2% FBS. The effect of purified IgG αAMRs on cell proliferation was examined by the 3-(4,5-dimethyl-2-thizolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, as described previously (10). After 6 d growth at 37°C in a humidified 5% CO_2/95% atmosphere, the dye and solubilization solutions were added from the Promega proliferation assay (Promega, Lyon, France). The BioTek Microplate manager plate reader and software (BioTek Instruments, Inc., Winooski, VT, USA) were used to determine the change in the number of viable cells from dye reduction measured by absorbance at 570 nm.

In vivo Matrigel studies and analysis

Female C57BL/6 mice were injected s.c. above the rectus abdominus with Matrigel (Becton-Dickinson, Le Pont de Claix, France), either combined with recombinant human VEGF 165 (R&D Systems, Minneapolis, MN, USA) and basic fibroblast growth factor (bFGF; R&D Systems) at 500 ng/ml or AM (Bachem) at 500 ng/ml or alone as a negative control. Twenty-four hours later, mice injected with Matrigel combined with AM were randomized into 4 groups and treated i.p. every 3 d with αAMRs (25, 100, or 500 μg) or preimmune serum (purified IgG, 500 μg). Three weeks later, a group of animals was sacrificed, and the Matrigel plugs were dissected and fixed in 4% paraformaldehyde (PFA) for histological analysis. Paraffin-embedded sections were processed and stained using hematoxylin and eosin (H&E). Eight central sections were photographed by investigators masked to sample identity. Immunohistochemical analysis was performed on paraffin-embedded sections with antibodies from Dako Inc. (Glostrup, Denmark) for CD31 (1:30), CD34 (1:40), CD45 (1:200), MOMA-2 (1:200), and smooth muscle α-actin (α-SMA; 1:80). Signal amplification utilized fluorochrome (Alexa 488 or Alexa 647)-conjugated secondary antibodies (1:250; Invitrogen Life Technologies).

The second group of animals was injected systemically into the lateral tail vein with 100 mg/kg FITC-dextran solution (molecular weight ~150,000; Sigma Chemical Co., Lyon, France) and allowed to circulate for 25–30 min. Before mice sacrifice, blood samples were collected by cardiac puncture and plasma was separated. The Matrigel plugs were resected, placed into tubes containing Dispase reagent (In Vitro Life Technologies, Cergy Pontoise, France), and homogenized. After centrifugation, the supernatant was saved for analysis of fluorescence. Fluorescence readings were obtained on an FL600 fluorescence plate reader (BioTek). Angiogenic response was expressed as a ratio of Matrigel plug fluorescence/plasma fluorescence.

In vivo tumor growth assessment

Athymic NMRI (*nu/nu*) nude mice were implanted with glioblastoma U87, colon HT-29, or lung A-549 tumor cells. Cell suspension of 2.5 × 10 cells was injected s.c. as described previously (10). Two weeks later, most tumors had grown to 200–300 mm^3, and mice were randomized into groups. Three independent experiments were performed, each with 24 animals in 3 groups. The treatment was i.p. injection, received every 3 d, of preimmune serum (330 μg of purified IgG) or αAMRs (330 μg of purified IgG/mouse containing 110 μg each of αCLR, αRAMP2, and αRAMP3 antibodies). Body weight, tumor size, and general clinical status were recorded every 2–3 d. Tumors were measured with a dial caliper, and volumes were determined using the formula width × length × height × 0.52 (for ellipsoid form).

Tumor immunohistochemical analysis

Tumor-bearing mice were injected with biotinylated lectin (Vector Laboratories, Burlingame, CA, USA) and heart perfused with 4% PFA, and blood vessels were stained with a streptavidin-conjugated fluorochrome (Alexa 488 or Alexa 647; Molecular Probes, Invitrogen Life Technologies). Thin (10-μm) or thick (100-μm) sections were incubated with anti-CD31 (1:30; Dako), anti-von Willebrand factor (anti-vWF) (1:400; Dako), or anti-αSMA (1:80; Dako), and subsequently with fluorochrome (Alexa 488 or Alexa 647)-conjugated secondary antibodies (Invitrogen Life Technologies). All tumors were excised, fixed in 10% (v/v) formalin, and processed for immunohistochemical analysis. Paraffin blocks were cut to 6-μm sections and stained with H&E for morphology evaluation. Immunohistochemistry was carried out using the Vectastain Elite ABC kit (Vector Laboratories). To assess programmed cell death, tissue sections were evaluated using Mab F7–26 to detect single-strand DNA (ssDNA; AbCys, Paris, France). For nonimmunofluorescence staining, detection was carried out using a DAB chromogen. Negative control slides were obtained by omitting the primary antibody.

Tumor vascular density

Quantitation of vessel count was performed as described previously (30). The blood vessels were counted randomly from nonnecrotic areas in each tumor section in an ×200 microscope field (1.0 mm; BH2;Olympus Tokyo, Japan), on

vWF-, CD31-, or αSMA-stained tissue sections. Vascular density was defined by averaging the number of vessels with lumen in ≥8 of the most vascular areas.

Statistical analysis

Data are expressed as means ± SE. Statistical analyses were performed by using the 1-way ANOVA followed by Fisher's protected least significant difference test (Statview 512; Brain Power Inc., Calabasas, CA, USA). Differences were considered significant at values of $P < 0.05$.

RESULTS

αAMRs inhibit recruitment of proangiogenic cells into Matrigel plugs *in vivo*

To evaluate whether αAMRs could inhibit angiogenic activities induced by AM in adult mice, we first used the *in vivo* Matrigel implant model to assess angiogenesis in response to specific factors in a noninflammatory setting. Mice were injected s.c. anterior to the abdominal rectus sheath with Matrigel alone or with Matrigel containing recombinant VEGF and bFGF (used as control), or AM peptide. Histochemical analysis of the Matrigel plugs revealed markedly increased cellularity induced by AM (**Fig. 1***Ac*) or VEGF-A and bFGF (Fig. 1*Ab*), whereas control Matrigel plugs exhibited cellularity of <2% (Fig. 1*Aa*). Matrigel plugs containing AM showed more vascular channels when compared with VEGF-treated plugs. Quantitative image analysis of endothelial cells (vWF positive) and pericyte cells (αSMA positive) demonstrated a 30–40% increase of these cell types in AM-treated plugs when compared with VEGF-treated plugs.

To demonstrate that αAMRs can directly inhibit angiogenesis *in vivo*, by inhibiting the recruitment of circulating AMR-positive (AMR^+) cells, Matrigel plugs supplemented with AM were injected s.c. into C57BL/6 mice, forming semisolid plugs. Twenty-four hours later, mice were treated by i.p. injection with increasing doses of αAMRs (Fig. 1*Ae–g*) or rabbit control IgG at 500 μg (Fig. 1*Ad*) every 3 d for a total of 21 d. Plugs taken from mice treated with αAMRs at 100 μg (Fig. 1*Af*) and 500 μg (Fig. 1*Ag*) had a markedly reduced cellularity of plugs. Matrigel plug angiogenesis was quantitated by measuring the uptake of FITC-dextran (~150,000) into plugs before their removal from mice. αAMR treatment significantly inhibited the amount of FITC-dextran in Matrigel plugs in a dose-dependent manner compared with rabbit control IgG (Fig. 1*B*). FITC-dextran uptake was decreased 9% for animals receiving 25 μg/dose of αAMRs, 19% ($P<0.05$ *vs.* control) for animals receiving 100 μg/dose, and 72% ($P<0.001$ *vs.* control) for animals receiving 500 μg/dose of αAMRs, a level marginally different from the negative control (Matrigel not supplemented with growth factors; Fig. 1*B*). Interestingly, immunostaining of sections of plugs with AM for vWF demonstrated the presence of vascular channels lined by endothelial cells (Fig. 1*Ca*). The same channels exhibited ensheathment by vascular smooth muscle cells, as assessed by α-SMA immunoreactivity (Fig. 1*Cb*). These data demonstrate that AM promotes recruitment of endothelial-like cells and pericyte cells and seems to play a role in cell-cell contacts that maintain transient microvessel stability (Fig. 1*Cc* and inset). Treatment with 500 μg of αAMRs decreased >90% of microvessel density, and very few microvessels can be observed in plugs after immunostaining with anti-vWF and anti-α-SMA antibodies.

To further assess the cell types recruited to the center of the Matrigel plug supplemented with AM, we performed immunohistochemistry using antibodies specific for endothelial cells (CD31), pericyte cells (α-SMA), macrophages/monocytes (MOMA2), mast cells (CD34), and leukocytes (CD45; **Fig. 2**). Significant numbers of cells recruited to the center of AM-loaded Matrigel plugs were AMR^+ cells, as demonstrated by coimmunostaining with CLR, RAMP2, and RAMP3 antibodies (Fig. 2). The expression of AMRs by these cells suggest that they are recruited specifically by AM in the Matrigel plugs.

It is of interest to observe that αAMRs raised against human epitope are able to recognize the mouse (m)CLR, mRAMP2, and mRAMP3 proteins. These data are not surprising since a high homology of the peptide sequence (94, 75, and 87% for CLR, RAMP2, and RAMP3, respectively) between human and mouse species was used to develop polyclonal antibodies (11). To detect mCLR, mRAMP2, and mRAMP3 proteins, membrane extracts (40 μg) were prepared from mouse tissues (kidney, brain, and lung) and subjected to Western blot analysis (**Fig. 3**). Samples from different tissues produced CLR as a distinct band of 48 kDa and a multimer, presumably heterodimers CLR/RAMP2 or CLR/RAMP3, at 73–76 kDa (Fig. 3). RAMP2 was seen as a monomer of 35 kDa, and multimers, presumably homodimers at 50 kDa and heterodimers CLR/RAMP2 or CLR/RAMP3 at 73 kDa, were seen (Fig. 3). RAMP3 was seen as a monomer of 19 kDa in all tissues, 35 kDa in kidney, 40 kDa in brain, and 38 kDa in lung, which may represent some heterogenously glycosylated monomer RAMP3. Multimer RAMP3, presumably as homodimers at 50 kDa and heterodimers CLR/RAMP2 or CLR/RAMP3 at 73 kDa, was seen in all tissues (Fig. 3). The specificity of our immune detection assay was confirmed by an antibody absorption control that eliminated the specific bands (Fig. 3). Taken together, these data demonstrate the expression of mCLR, mRAMP2, and mRAMP3 in several mouse tissues and the ability of the polyclonal antibodies generated against the extracellular domains of human (h)CLR, hRAMP2, and hRAMP3 to detect the mouse proteins.

Effect of αAMRs on proliferation of human cancer cell lines

Previously, we have demonstrated the expression of CLR and RAMP2 in U87 cells but not the L1 receptor

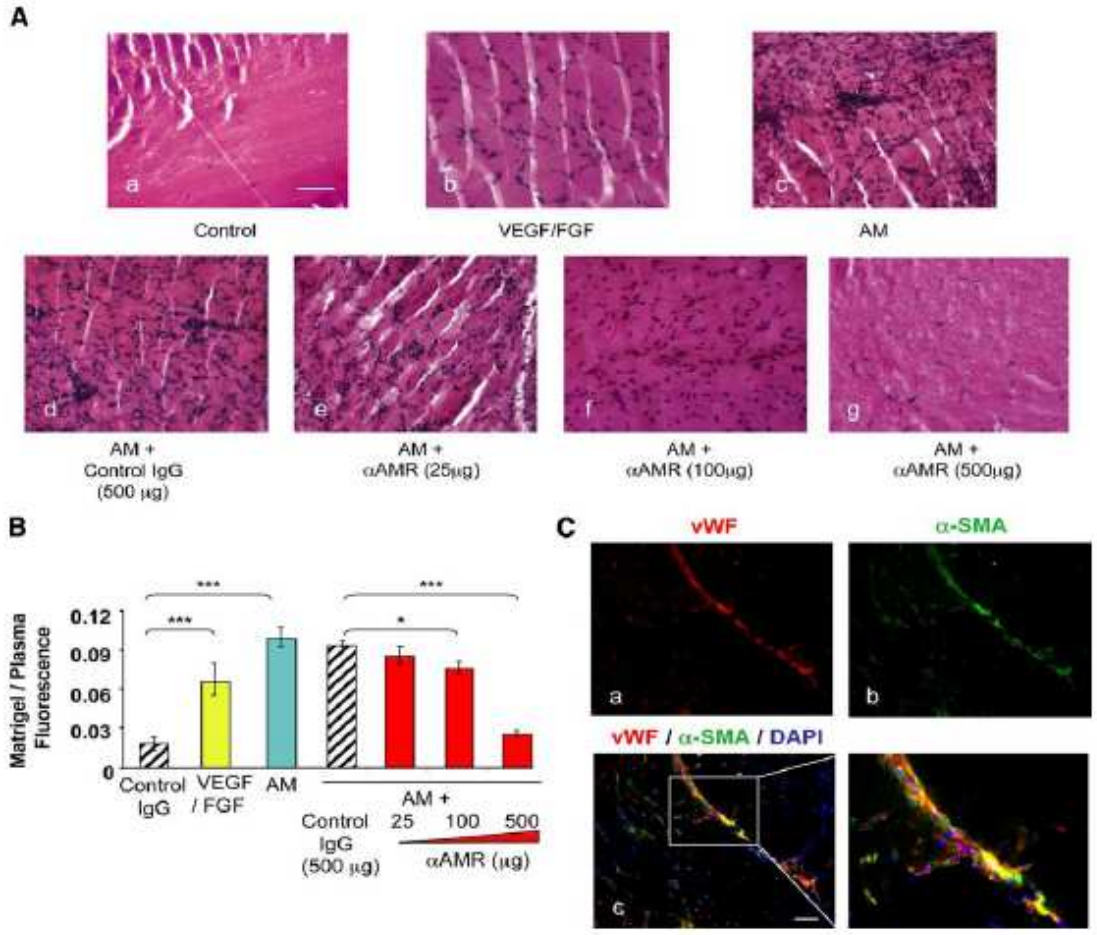

Figure 1. αAMRs inhibit cell recruitment into Matrigel plugs. *A*) C57BL/6 mice were injected s.c. at the abdominal midline with 0.5 ml of growth factor-depleted Matrigel containing VEGF-165 and FGF (500 ng/ml; *b*) or AM (500 ng/ml; *c*) or no additional growth factor (control; *a*). αAMRs (*e–g*) or control IgG (*d*) were administered i.p. to C57BL/6 mice with plugs containing AM every 3 d, starting 24 h after Matrigel injection, for 21 d. Microphotographs of histochemical-stained Matrigel sections for H&E are shown. Scale bar = 200 µm. *B*) After 21 d of treatment, mice were injected i.v. with FITC-dextran (~150,000); Matrigel plugs were removed, and the volume of new blood vessels was assessed by measurement of intravascular FITC-dextran content (normalized to FITC-dextran in the circulating plasma). Values are averages ± SE of 6 animals. $^{*}P < 0.05$; $^{***}P < 0.001$. *C*) Animals with Matrigel plugs containing AM were sacrificed a 3 wk later. Matrigel plugs were isolated and fixed with formalin, paraffin embedded, and sectioned for immunohistochemical analysis. Sections were evaluated by immunofluorescence for vWF (*a*) and α-SMA (*b*) and demonstrated the presence of vessel structures characterized by a tight interactions between endothelial-like cells and pericytes (*c*). Bottom right panel is a closeup of the inset in *c*. DAPI-stained nuclei are blue. Scale bar = 100 µm.

(10). Barely detectable expression for the L1 receptor can be found in HT-29 and A549 cells (not shown). αAMRs strongly blocked AM-labeled [^{125}I-AM] binding to the cell membranes in a dose-related manner, suggesting that heterodimers CLR/RAMP2 and CLR/RAMP3 are present at the plasma membrane of the U87, HT-29, and A549 cell lines. αAMRs at 10, 20, and 40 µg/ml showed 25, 45, and 80% inhibition of specific binding, respectively. Previously, we reported that AM is involved in U87 cell proliferation by the autocrine/paracrine growth loop (10). To determine whether αAMRs can inhibit cell growth *in vitro* of different cell lines, U87 cells, HT-29 cells, and A549 cells were exposed to increasing concentrations of αAMRs at 10, 35, and 70 µg/ml, and the effect on proliferation was assessed by cell counting or MTT assay. αAMRs inhibit U87 cell proliferation in a dose-dependent manner and reached 23% ($P<0.05$), 41% ($P<0.01$), and 57% ($P<0.001$), respectively, by 6 d of treatment (**Fig. 4A**). HT-29 cells showed a higher degree of inhibition, reaching 30% ($P<0.039$), 62% ($P<0.0013$), and 75% ($P<0.0011$; Fig. 4*B*). Surprisingly, A549 cells did not show any decrease in proliferation despite the expression of CLR, RAMP2, and RAMP3 proteins (Fig. 4*C*). The same data were

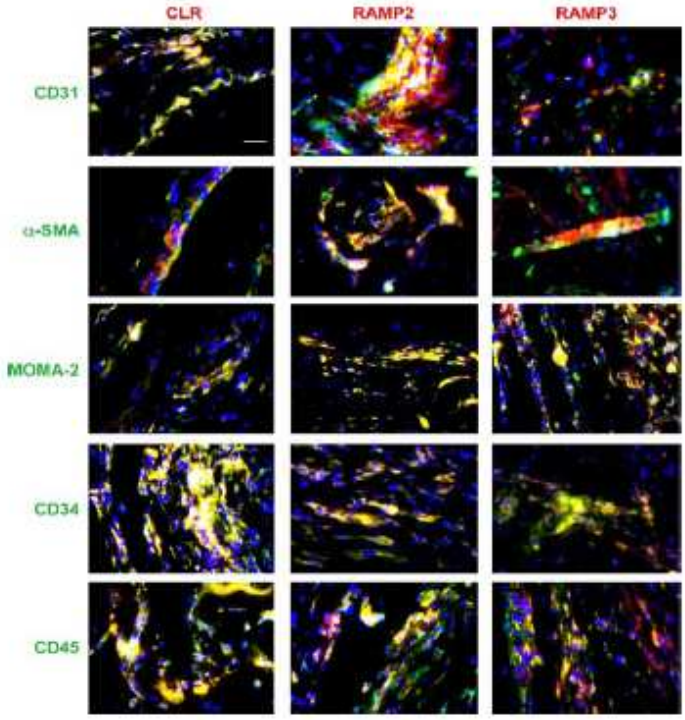

Figure 2. AM induces recruitment of different cell types in an *in vivo* Matrigel assay. Immunofluorescence for CD31, α-SMA, MOMA-2, CD34, CD45 (green), and AMRs (CLR, RAMP2, and RAMP3; red) was assessed. Costaining (yellow) demonstrates the expression of AMRs by a great number of infiltrating endothelial-like cells, pericytes, and macrophages/monocytes. DAPI-stained nuclei are blue. Scale bar = 40 μm.

obtained with anti-AM antibody treatment for A549 cells (not shown), suggesting that AM is not involved in A549 cell growth *in vitro*. In contrast, the treatment with boiled αAMRs (not shown) or control IgG of irrelevant specificity at 70 μg/ml (Fig. 4*A*, *B*) showed no inhibition of cell growth. Together, these data suggest that αAMRs are able to recognize the epitope on AMRs preventing AM from activating the autocrine/paracrine growth loop.

αAMRs inhibit growth of human tumor xenografts

Treatment of healthy mice (n=8) with αAMRs did not affect body weight, did not cause any obvious organ pathology on inspection and histological analysis, did not alter the clinical chemistry or hematological blood profile, and did not increase proteinuria. The antitumor activity of αCLR, αRAMP2, and αRAMP3 was tested either separately or combined in the U87 xeno-

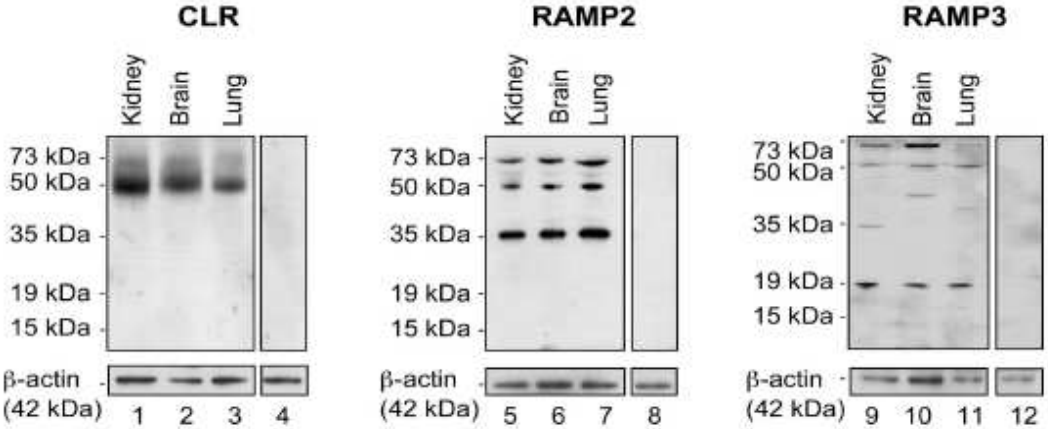

Figure 3. Expression of CLR, RAMP2, and RAMP3 in mouse tissues. Membrane extracts (40 μg) prepared from mouse tissues (kidney, brain, and lung) were separated on 10% SDS-PAGE, transferred to Hybond-C membrane, and immunoblotted using antibodies generated against CLR, RAMP2, and RAMP3 peptides, as described previously (11). Staining for all samples was blocked by the addition of 20 μg/ml CLR, RAMP2, and RAMP3 peptides (lanes 4, 8, and 12). To assess the amount of proteins loaded for each sample, immunoblot analysis was performed with anti-β-actin.

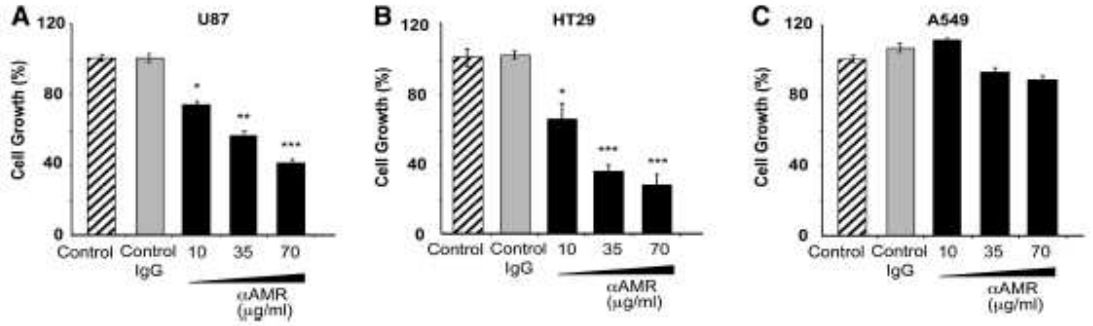

Figure 4. Effect of αAMRs on the growth of human cancer cell lines *in vitro*. Proliferation assays, U87 cells (*A*), HT-29 cells (*B*), and A549 cells (*C*) were seeded at the density of 2×10^3/well in 12 multiwell plates in the presence of EMEM supplemented with 2% FBS. Increasing concentrations of αAMRs were added. As controls, boiled αAMRs (not shown) or rabbit control IgG (70 μg/ml) of irrelevant specificity were used. For each treatment, 6 wells were prepared for MTT assays or exposed to trypsin, and cells were counted in a coulter counter (not shown). Values are means ± SE of 4 independent experiments. $*P<0.05$; $**P<0.01$; $***P<0.001$.

graft model, which exhibits aggressive tumor growth kinetics. Untreated control U87 xenografts reached a size of >2000 mm^3 within 18 d. By d 30, mice become moribund in the control group, and the experiment was terminated. We first performed experiments to determine the capacity of each antibody to inhibit tumor growth *in vivo*. A slight decrease in tumor growth was observed using αRAMP2 or αRAMP3 as compared with control IgG treatment (1 mg/wk; **Fig. 5***A*). Better results were obtained with αCLR at 1 mg/wk as compared with all groups (Fig. 5*A*). However, the αAMRs at 1 mg/wk demonstrated a clear inhibition of tumor growth (Fig. 5*A*). Subsequently, the combined antibodies (αAMRs) were used in the *in vivo* experiments. αAMR treatment inhibited tumor growth in a dose-dependent manner compared with rabbit control IgG (not shown). The maximal effective dose in this tumor model was 330 μg of αAMRs administered by i.p. injection every 3 d ($P<0.001$ *vs.* control IgG). No signs of toxicity such as weight loss in response to αAMR treatment were observed. Pathological examination of tissues from treated mice also appeared normal.

To further examine the effect of αAMR therapy on human tumor growth, athymic nude mice bearing established glioblastoma, colon, or lung xenograft tumors (>200 mm^3) were treated systemically with αAMRs (Fig. 5*B–D*). αAMR treatment significantly inhibited the growth of subcutaneous U87, HT-29, and A549 tumors compared with control groups ($P<0.001$; Fig. 5*B–D*). After 20 d of treatment, a group of animals was sacrificed, and tumor size and vascularity were assessed. Tumors derived from

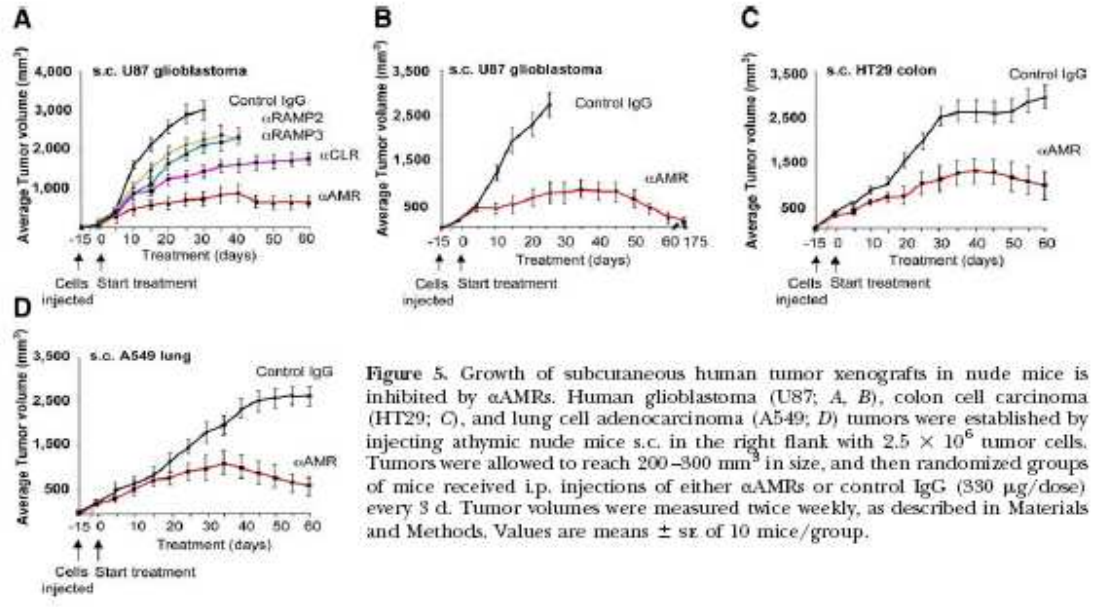

Figure 5. Growth of subcutaneous human tumor xenografts in nude mice is inhibited by αAMRs. Human glioblastoma (U87; *A, B*), colon cell carcinoma (HT29; *C*), and lung cell adenocarcinoma (A549; *D*) tumors were established by injecting athymic nude mice s.c. in the right flank with 2.5×10^6 tumor cells. Tumors were allowed to reach 200–300 mm^3 in size, and then randomized groups of mice received i.p. injections of either αAMRs or control IgG (330 μg/dose) every 3 d. Tumor volumes were measured twice weekly, as described in Materials and Methods. Values are means ± SE of 10 mice/group.

αAMR-treated animals appeared pale or transluscent, with diminished vasculature and permeability (not shown). In contrast, large tumors with extensive vascularization were observed in control groups. The mean tumor weights in the control and in the αAMR-treated groups were 3.1 *vs.* 0.8 g, 2.5 *vs.* 0.9 g, and 3.4 *vs.* 1.1 g for U87, A549, and HT-29 xenograft tumors, respectively, at 25 d (U87) and 40 d (A549; HT-29) of treatment. No relapse of tumor growth was observed with continued administration for up to 10 wk in the U87 xenograft model. Withdrawal of αAMR treatment for up to 200 d resulted in no regrowth of all tumors.

Inhibition of AMR signaling depletes endothelial cells and pericytes in tumors

If AM is able to promote a recruitment of pericytes that are AMR$^+$, then using a neutralizing αAMR should lead to an overall reduction of tumor pericytes. To elucidate further the mechanisms involved in the tumor regression after treatment with αAMRs, tissue histopathology assessment was undertaken. Histological examination of U87, HT-29, and A549 tumors removed from animals after αAMR treatment for 3 wk showed a markedly decreased vessel density when compared with the control IgG-treated group, as demonstrated for U87 tumors (**Fig. 6*A, B***). Tumor-bearing mice that were injected i.v. with biotinylated lectin and stained with a streptavidin-conjugated fluorochrome demonstrated that αAMR-treated tumors were significantly less vascular (Fig. 6*Ab*) than control tumors (Fig. 6*Aa*). The same data were obtained by immunostaining of tumors with antibodies for CD31 (Fig. 6*B*) and vWF (not shown). Immunostaining analysis with CD31 and αSMA antibodies demonstrates a clear inhibition of blood vessel density by αAMR treatment of A549 tumors (see Supplemental Fig. S1) and HT-29 (not shown). Quantification of CD31-stained endothelial cells demonstrate a clear decrease of CD31 cells in αAMR-treated tumors when compared with control IgG-treated tumors ($P<0.001$; Fig. 6*C*), suggesting that endothelial cells need a contact with a perivascular cells such as pericytes for survival. Quantitative evaluation of the number of pericytes by antibody staining (green) revealed an overall reduction of pericytes of 85–90% ($P<0.001$; Fig. 6*D*), compared with pericytes in control IgG-treated tumors (Fig. 6*A, B*). Tumors treated with rabbit control IgG, however, did not exhibit any pericyte detachment. It is important to note that neutralizing αAMRs did not affect the normal vasculature nor the pericyte numbers or attachment in normal organs such as kidney, lung, and liver (not shown). Taken together, these data demonstrate that αAMRs reduce tumor growth, at least in part, by inhibiting tumor angiogenesis.

To determine the consequences of αAMR treatment on tumor vessel area, tumor xenografts were excised at 6, 16, and 25 d of treatment and immunostained with vWF antibody (**Fig. 7*A***). After 25 d of treatment, the mean vessel area was significantly decreased in the αAMR-treated tumors when compared with control

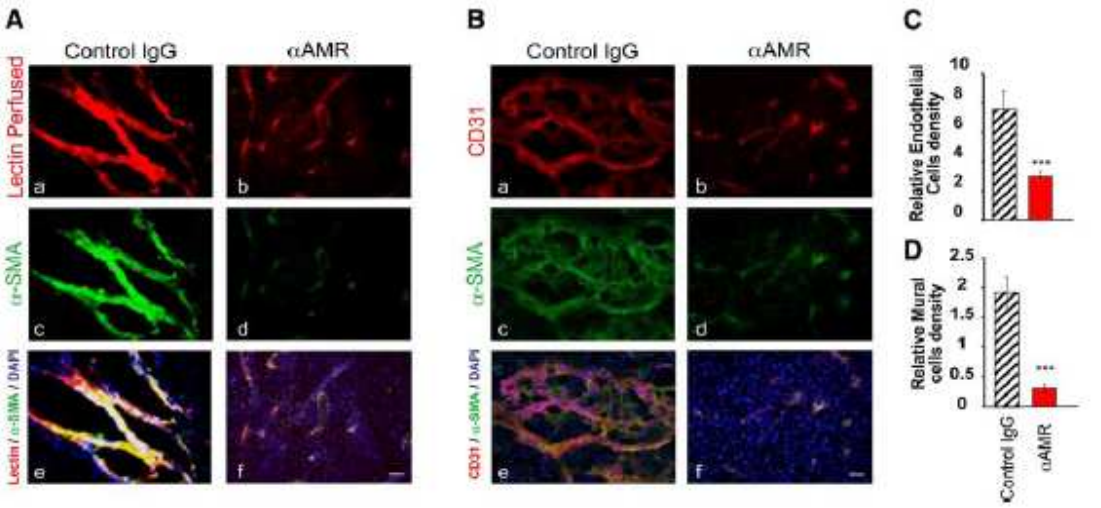

Figure 6. Depletion of endothelial cells and pericytes in αAMR-treated tumors. U87 cells (2.5×10^6) were implanted s.c. into athymic (*nu/nu*) mice. After 15 d, when primary tumors were 300–400 mm^3 in size, animals were randomly divided into two groups and treated with αAMRs or rabbit control IgG of irrelevant specificity. *A, B*) Representative images of tumors from control and αAMR-treated animals. Mice were first anesthesized and injected i.v. with biotinylated lectin and then heart-perfused with 4% PFA; tumor sections (50 μm) were incubated with streptavidin-conjugated fluorochrome to reveal blood vessels (*A*). Alternatively, to visualize functional blood vessels in tumors, tumor sections (50 μm) were evaluated by immunofluorescence for CD31, a marker to detect endothelial cells, and α-SMA, a marker for mature pericytes (*B*). DAPI-stained nuclei are blue. Scale bars = 100 μm. *C, D*) Quantitative assessment of cell density of cells that stained positive for CD31 (*C*) or α-SMA (*D*) was conducted through a microscope. NIH Image 1.62 software was used for analysis. Values are means ± SE; $n = 6$. $^{***}P < 0.001$.

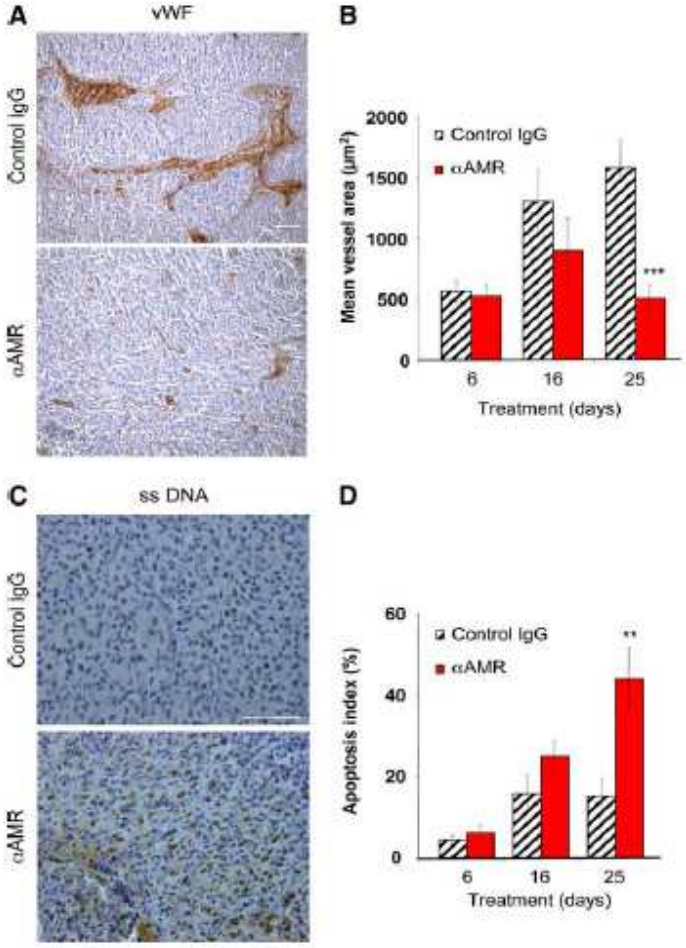

Figure 7. Effect of αAMRs on a human glioma xenograft. U87 cells (3×10^6) were implanted s.c. into athymic (*nu/nu*) mice. After 6, 16, and 25 d of treatment with αAMRs or IgG control, animals were sacrificed, and subcutaneous glioma tumors were harvested. *A*, *C*) Microphotographs of immunohistochemical-stained tumor sections for vWF and ssDNA in control and αAMR-treated tumors. *B*) Quantitative assessment of cell density of cells that stained positive for vWF and measurement of mean vessel area (μm^2) were conducted through a microscope. NIH Image 1.62 software was used for analysis. Values are means ± SE; $n = 8$. *D*) Percentage of cells undergoing apoptosis was determined by staining for ssDNA with Mab F7–26 antibody. Values are means ± SE; $n = 6$. $^{**}P<0.01$; $^{***}P<0.001$.

tumors ($P<0.001$; Fig. 7*B*). Accordingly, and despite the fact that the apoptosis labeling revealed by immunostaining of ssDNA with Mab F7–26 antibody is heterogeneous among the tumors, the apoptotic index of the αAMR-treated tumors increased with treatment (Fig. 7*C*) and reached a significant increase of ~3-fold at d 25 when compared with control tumors ($P<0.01$; Fig. 7*D*).

DISCUSSION

Neovascularization is a common attribute of tumors, and a wealth of functional studies (7, 31) support the proposition that blood vessels are crucial for the formation, tumor growth, and dissemination of cancer cells. Solid tumors are not just composed of malignant cells but are complex tumor-microenvironment interactions involving many cell types, including a wide range of hematopoietic and myeloid cells. AM interacts with its receptors (CLR/RAMP2 and CLR/RAMP3), which are expressed by a broad range of cell types, including cancer cells, endothelial cells, mural cells, hematopoietic cells, and myeloid cells. By recruiting and entrapping diverse proangiogenic cells and by promoting *in situ* neoangiogenesis, AM clearly seems to play a key role in neovascularization and/or vessel stabilization. Our results demonstrate that AM is highly effective in recruiting endothelial cells and pericytes (as stained positive for α-SMA and desmin; not shown), an important step in the stabilization and maturation of new vasculature, into the Matrigel matrix, as many vascular channels became ensheated with vascular smooth muscle cells in response to AM. Localized expression of the AMR on a subset of endothelial, pericyte cells in the uninjured dermis suggests that AM may act directly on this resident population of cells, with the possibility of recruitment of blood-borne vascular precursors to the dermis. Indeed, the cellular accumulation of the Matrigel plugs in response to AM is likely to reflect a significant component of blood-borne cells that are capable of differentiating *in situ* into vascular channels. These data demonstrate that AM can recruit AMR^+ proangiogenic cells to promote

the assembly of new vessels and support a long-lasting augmentation in vessel integrity and stability. These observations are further strengthened by the observation that AM-mediated angiogenesis is inhibited on neutralization of AM by a specific antibody (not shown).

In this study, we demonstrated that blockade of the CLR/RAMP2 and CLR/RAMP3 receptors by systemic administration of the combined antibodies, αAMRs, inhibits angiogenesis in an *in vivo* model and the growth of several human tumors. The rationale for using αAMRs is that this approach allows for a highly specific and potent means to block AMR function. We demonstrated that addition of AM to Matrigel significantly enhanced plug neovascularization, which was effectively inhibited by systemic injection of αAMRs in a dose-dependent manner. To examine tumor growth in response to αAMR therapy, we chose the rapidly tumorigenic glioblastoma U87 cells, lung tumor A549 cells, and colon tumor HT-29 cells. The treatment of human xenograft-bearing mice with αAMRs consistently resulted in tumor regression, suggesting that the tumor is most susceptible to αAMR therapy. Since U87 and HT-29 cell growth *in vitro* is inhibited by αAMRs, the inhibition growth of the corresponding tumor xenografts *in vivo* could be a result of a combined effect on tumor cell growth associated with the effect on tumoral neoangiogenesis. By contrast, A549 cells treated with αAMRs showed no growth inhibition *in vitro* but demonstrated a clear decrease for its corresponding xenograft *in vivo* on αAMR treatment. The *in vivo* growth inhibition is caused by a direct and isolated effect of αAMRs on tumor vasculature. The immunostaining with anti-vWF and anti-α-SMA antibodies demonstrated a clear decrease of endothelial and pericyte cells in αAMR-treated A549 tumors when compared with control tumors (Supplemental Fig. S1). Interestingly, the density of vessels with lumen decreased remarkably (Supplemental Fig. S1).

It is well known that subcutaneous tumors have a different vascularity than orthotopic tumors; for the purpose of evaluating the effect of αAMRs in orthotopic models, we developed orthotopic xenografts of glioblastoma U87 and human prostate cancer of androgen-independent (DU145) cells. Treatment with αAMRs or αAM caused a clear inhibition of tumor growth characterized at the histological level by a vascular disruption with decreased microvessel density and increased tumor cell apoptosis, greater than the effects obtained with subcutaneous tumors (unpublished results). Taken together, the data obtained with both models highlight the key role of the AM system (AM and its receptors) in tumor growth *in vivo*.

It should be noted that no relapse was ever observed with continued administration of αAMRs for up to 60 d. Withdrawal at d 70 of αAMR treatment in the U87 xenografts resulted in no regrowth of tumors for up to 200 d. The results of the present study are consistent with the data obtained with the αAM *in vivo* (10) or AM antagonist (AM_{22-52}; ref.28) and highlight the AM-AMR (mainly CLR/RAMP2/RAMP3) axis as a target for new antiangiogenic and antitumor therapies. Our data are supported by studies (21, 22) in which *CLR* and *RAMP2* null mice die *in utero* due to vascular abnormalities. However, although the L1 receptor is barely detectable in our models, studies (25) are in progress to evaluate the involvement of the L1 receptor in tumor cell growth *in vitro* and *in vivo*, as it was recently reported for pancreatic cancer cells.

The reduction in microvessels occurred rapidly, *i.e.*, after 8–10 d of treatment, with αAMRs and reached a peak after 14–25 d of treatment. The loss of microvessels within treated tumors suggests that AM stimulation of CLR/RAMP2/RAMP3-expressing tumor vasculature acts as a survival mechanism for proliferating tumor endothelium. The high increase in endothelial cell apoptosis (not shown), when pericytes were depleted from blood vessels, further strengthened the function of tumor pericytes in protecting endothelial cells, probably by expressing potent endothelial survival factors. The data support the proposition that AMR^+ perivascular cells associated with the tumor vasculature are functionally important for the maintenance of tumor blood vessels, adding another constituent cell type in tumors to the list of anticancer targets. Association of AMR^+ perivascular cells with the tumor endothelial cells is ostensibly maintained by the expression of AM ligand in the endothelial cells, establishing a paracrine homeostatic signaling circuit analogous to the situation of PDGF ligands and PDGFR during embryonic development of certain tissue vascular beds (32, 33). The expression of AM by endothelial cells and the expression of AMR by pericytes point to the importance of AM signaling in perivascular cells for sustaining the tumor vasculature *via* association with endothelial cells.

It should be noted that we observed no toxicity associated with long-term treatment of tumor-bearing animals. Autopsy of αAMR-treated adult mice revealed no abnormalities of physiological vasculature in the organs of these mice, including the heart, liver, kidney, lung, and spleen. These findings are important, since low levels of CLR, RAMP2, and RAMP3 expression are expected to be present on the endothelium of the majority of normal tissues. In normal adult tissues, the turnover of endothelial cells is very low (years) except in those tissues that undergo normal angiogenic processes. Therefore, the lack of toxicity observed during αAMR therapy may be due to the limited dependence of the resting endothelium for CLR/RAMP2 and CLR/RAMP3 stimulation. In contrast, tumor angiogenesis and tumor growth are expected to be more dependent on up-regulation and function of AMR on tumor vasculature and the recruitment of different blood borne cells necessary to contribute to tumor growth and thus more susceptible to αAMR blockade. The lack of toxicity associated with αAMR treatment can also be attributed to the high specificity on an antibody antagonist. It is conceivable that

AMR blockade will have an impact on angiogenesis associated with reproduction, wound healing, or other normal processes involving angiogenesis, such as bone formation. Studies are currently ongoing in our laboratory to address these questions.

The histological examination of tumors from αAMR animals showed a decrease in microvessel density with a 85% reduction of pericyte numbers (α-SMA- or desmin-stained cells) within the tumor, confirming the critical role of AMR signaling in pericyte activation and/or recruitment. These results support the hypothesis that pericytes in tumors are formed *de novo* by maturation of undifferentiated perivascular cell progenitors recruited to the newly formed vasculature from bone marrow or from a preexisting pool of pericytes. Abrogation of AMR signaling reduced the number of activated pericytes in tumors, whereas quiescent pericytes in normal tissues were not affected. Our results from αAMR -treated tumors clearly underline the functional importance of tumor pericytes. These results imply that tumor pericytes, albeit less abundant or more loosely attached, still regulate vessel integrity, maintenance, and function.

Finally, preliminary results in our laboratory showed an inhibition of intratumoral macrophage infiltration and myeloid precursor cells, suggesting that AM might be involved in the recruitment of tumor-associated macrophages *in situ*. These data are supported by the Matrigel plug *in vivo* experiment, which demonstrated that macrophage/monocyte cells (myeloid-derived cells) were among the cell types recruited by AM. Recently, it was reported by Zudaire *et al.* (34) that AM is chemotactic for mast cells *in vitro* and might be involved in mast-cell recruitment to tumors. Although there is currently no evidence for this *in vivo*, experiments using Matrigel plugs *in vivo* with AM showed a great infiltration of cells with the CD34 cell-surface marker of mast cells in mice (35), suggesting that AM might be able to promote recruitment of mast cells into tumors.

In summary, we demonstrated that αAMR antibodies effectively inhibit different cell type recruitment, angiogenesis, and tumor growth *in vivo*. These results suggest that blockade of the AM receptors may be a useful strategy for treatment of human cancer. Furthermore, the use of αAMR therapy in combination with conventional cytotoxic, radiation, immunotherapy, or other antiangiogenic agents may improve the efficacy of these anticancer therapies.

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PUBLICATION 3

Expression of Adrenomedullin and Adrenomedullin receptors in tumor glial cells and vascular derived cells from malignant human gliomas.

Carine Tesic[1,2], Christine Delfino[1,2], Yolande Berthois[1,2], Philippe Metellus [1,2,3], Vincent Achard[4], L'Houcine Ouafik[1,2,5], Françoise Boudouresque[1,2].

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Carine Tesic[1,2], Christine Delfino[1,2], Yolande Berthois[1,2], Philippe Metellus [1,2,3], Vincent Achard[4], L'Houcine Ouafik[1,2,5], Françoise Boudouresque[1,2].

[1] Inserm, UMR 911 - CRO2, Marseille, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 05, France;

[2] Aix-Marseille Université, Faculté de Médecine, Marseille, F-13005, France;

[3] Service de Neurochirurgie, Assistance Publique Hôpitaux de Marseille, CHU Timone

[4] Inserm, UMR 626, Marseille, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 05, France;

[5] Laboratoire de Transfert d'Oncologie Biologique [Assistance Publique Hôpitaux de Marseille, CHU Nord]

Abstract

Malignant gliomas and specially glioblastoma multiforme are the most frequent and devastating brain tumors in adults, and their growth is angiogenesis-dependent. The formation of abnormal, dysfunctional tumor vasculature and glioma cell invasion along white matter tracts are believed to be major components of the inability to treat these tumors effectively. AM (Adrenomedullin) is a multifunctional peptide vasodilator that transduces its effects through calcitonin receptor-like receptor/receptor activity modifying protein-2 and -3 (CLR/RAMP2 and CLR/ RAMP3). Previously, we demonstrated that AM induced angiogenic-related effects on endothelial cells HUVEC *in vitro* and suppressed growth of human glioblastoma U87 tumor xenografts in mice inhibiting their angiogenesis. In this study, we first confirm AM and its receptors (CLR, RAMP2, RAMP3) expression, in cultured glioblastoma tumor glial cells (TGCs) and glioblastoma associated vascular cells (GVCs), from patient's resected tumors. RT-PCR analysis reveal the same pattern of ARNm expression in both cell types and higher expression of CLR and RAMP2 in GVCs compared with TGCs. Nevertheless, GVCs secrete ~ 3 fold higher amounts of AM compared with TGCs, suggesting that AM is a good target to affect tumoral vasculature. Moreover, we hightlight the autocrine and paracrine AM induction of TGC and GVC motility, invasion and the specific contribution of AM secreted by TGCS to the arrangement and organization of VGCs into a meshwork of capillary-like tubular structures. These results from patients' cells sustain the potential use of AM and its receptors as anti-angiogenic and anti-invasive

therapeutic targets.

Introduction

Glioblastomas are highly malignant brain tumors with a median patient survival time of 9 to 14 months. They are characterized by rapidly dividing cells, invasion into normal brain, and a high degree of vascularity. The histological grade of malignant gliomas, based on vascular proliferation, endothelial cell hyperplasia and microvessels counts has been shown to be inversely related to prognosis (1, 2). Glioblastoma multiforms are characterized by a dilated, tortuous, disorganized and leaky vasculature. Tumor vessels are structurally and functionally abnormal, with defective endothelium basement membrane and pericyte coverage (3). Due to their critical role for tumor growth, tumor blood vessels are recognized as potential antitumor drug targets (4) and their abnormalities open up the possibility of targeting tumor vessels separately from the vessels of the surrounding normal tissue (5). Evidence has accumulated that many endogenous peptides play an important regulatory role in angiogenesis by modulating endothelial cells behaviour. These peptides include Adrenomedullin (AM) that belongs to a family of regulatory peptides including calcitonin, amylin, and calcitonin gene-related peptide (6) (CGRP). AM is a widely distributed multifunctional peptide ranging from inducing vasorelaxation to acting as a regulator of cellular growth (7). AM is expressed in a variety of malignant tissues and was shown to be mitogenic for human cancer cell lines including lung, breast, colon, glioblastoma, pancreas and prostate lineages *in vitro* (8-10). Cell lines of different origins including glioblastoma cells express AM and its receptors, indicating that AM may function as an autocrine growth factor for tumoral cells (11, 12). It has been reported that AM is able to stimulate the proliferation of a number of human cancer cell lines *in vitro* (13), to be involved in the multistep process of angiogenesis *in vitro* and *in vivo* (14, 15), and to inhibit apoptosis in endothelial cells as well as in tumoral cells (16-20). Our group has demonstrated that AM expression is significantly higher in malignant glioblastomas and grade II astrocytomas when compared to low grade brain tumors and non tumoral brain tissue. Interestingly, *in situ* analysis of tumor specimens undergoing neovascularization demonstrated that the production of AM is specifically induced in a subject of GBM cells distinguished by their immediate proximity to necrotic foci (presumably hypoxic régions), suggesting a hypoxic induction of AM expression in GBM (21).

AM tranduces its effects through the G-protein coupled receptor calcitonin receptor-like receptor (CLR), with specificity for AM being conferred by the receptor activity modifying protein-2 (RAMP2) and -3 (RAMP3) (22). The ability of CLR/RAMP2 and CLR/RAMP3 to respond with high affinity to AM implies the existence of two molecularly distinct AM receptors referred to as AM_1 and AM_2 receptors, respectively (23). Recently, we demonstrated that blockade of the CLR /RAMP2 and CLR/RAMP3 receptors by systemic administration of the anti-AMR antibodies, inhibits angiogenesis in an *in vivo* model, and the growth of several human tumor xenografts *in vivo* (13). Furthermore, treatment with a neutralizing anti-Adrenomedullin antibody decreases the proliferation of glioma cells *in vitro*, and suppresses the growth of human glioma xenografts *in vivo* by disrupting the tumor xenografts vascularisation (9). Taken together, these data suggest either strongly that AM produced in tumor foci by tumor cells or cells of microenvironment might play a key role to have a stable and functional vascularization during tumor growth (8).

Glioblastoma is one of the most vascularized tumors, the aim of this work is to analyse the expression of AM system (AM and its receptors CLR, RAMP2 and RAMP3) in the glial tumor cells and vascular-derived gliblastoma cells. We investigated the effect of AM in the multistep process of neovascularization, including migration, invasion and morphogenesis of vascular derived glioblastoma cells.

Materials and Methods

Primary cultures of tumor glial cells (TGCs) and glioma associated vascular cells (GVCs).

Tumor tissue from patients operated on for glioblastoma at the Department of Neurosurgery, CHU Timone, Marseille, France, were collected at the time of surgery. All the tissue procurement protocols were approved by the relevant institutional committees (University of Aix-Marseille) and were undertaken Under informed consent of each patient or relatives. Diagnosis and grading of all tumors were certified by independent neuropathological examination. We obtained primary cell cultures from six surgical specimens classified into WHO grade IV. Brain samples collected at the term of surgery were processed by dissection into small pieces (< $1mm^3$) in medium RPMI

1640 (Invitrogen life Technologies Inc. Paris, France) supplemented with 2% FBS, and disrupted mechanically. Tumor vascular cells were isolated with a modified protocol described by (24). Tumor microvessels were enriched after centrifugation 10 minutes at 1000 × g in RPMI containing 15% Dextran (Sigma-Aldrich Chemistry). Cellular pellets of microvessels were subsequently dissociated with enzymatic digestion using collagenase at 1mg/ml (Worthington) in RPMI 1640 containing 2% FBS at 37°C. Microvascular cells were plated on collagen I pre-coated dishes and maintained in EBM2 medium supplemented with endothelial cell growth supplements EGM2 (Clonetics Lonza), penicillin (50µg/ml), streptomycin (50µg/ml) in a humidified incubator at 37°C with 5% CO_2, 95% air. The medium was replaced every 2 days.

Tumor glial cells located at the top of the dextran gradient were harvested, washed in RPMI 1640 and subsequently dissociated in collagenase (10µg/ml) (Sigma-Aldrich Chemistry) for one hour at 37°C. Cells were washed and taken in DMEM/F12 medium (Invitrogen life Technologies Inc.) supplemented with 10% FBS, penicillin (50µg/ml) and streptomycin (50µg/ml) and kept in a humidified incubator as described above. Cells were grown to confluence with medium change twice a week.

Immunocytochemistry

Immunohistochemical staining was performed on TGCs seeded on 2% gelatine coated two chambers labtek (LabTek chamber glass slide, Nunc) and on GVCs seeded on collagen I coated two chamber labtek. The cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature and washed three times with phosphate buffer (pH 7.4). After blocking with 3% of normal sheep serum (NSS) for 30 minutes, the cells were incubated with primary antibodies overnight at 4°C. Immunostaining for AM, CLR, RAMP2 and RAMP3 was performed using specific rabbit serum as described (10, 13). Optimal dilutions for antibodies were as follow: anti-AM antibody (1/1500), anti-CLR antibody (1/3000), anti-RAMP2 antibody (1/500), anti-RAMP3 antibody (1/2000).

Immunocytochemical analysis were performed using antibodies against GFAP (1:500), CD105 (1:600); Tuj-1 (1:500) and smooth muscle α-actin (α-SMA 1:100) from Dako Inc. (Glostrup, Denmark). Signal amplification utilized fluorochrome (Alexa 488 or Alexa 568) conjugated secondary antibodies (1:250), (Invitrogen life Technologies

Inc.). The cells were visualized with a Nikon fluorescence microscope using appropriate filters. Negative control slides were obtained by omitting the primary antibody.

Real-time quantitative RT-PCR

Total RNA was extracted from TGCs and GVCs (25) and reverse transcribed to cDNA as previously described (12). Real-time quantitative RT-PCR method was used to accurately detect the changed in the expression of the selected genes as previously described (9). The levels of human mRNAs encoding AM, CLR, RAMP2, RAMP3 and 18 rRNA were determined using the forward and reverse primers and conditions as previously described (10, 12) (Table 1). Quantitative mRNA expression data were acquired and analysed using LC 480 PCR System (Roche Diagnostics, Meylan, France).

Western blot analysis

Cell pellets were prepared and processed for Western blot analysis as described (9). Proteins were resolved by 12% SDS-PAGE and electrophoretically transferred to Hybond-C membrane (GE Healthcare, Europe GmbH) for 1 hour at 1mA/cm^2. Hybond-C strips were blocked in PBS containing 5% non fat dry milk and incubated overnight in 1/250 dilution of rabbit anti-serum anti-human CLR, RAMP2 and RAMP3 previously described (14) and washed three times in PBS. A polyclonal anti-β-actin antibody (Cytoskeleton Inc / Tebu-Bio, le Perray en Yvelines, France) was used at 1/1000 dilution. Signals were revealed using an enhanced chemiluminescence kit (ECL kit, GE HEALTHCARE, Europe GmbH, Orsay, France). Specificity control consisted of a duplicate membrane incubated in antigen-preadsorbed (10 nmol/ml) anti-serum.

AM radioimmunoassay

Medium from primary cell cultures were prepared to perform radioimmunoassay (RIA) of AM as previously reported (9).

ELISA

To determine VEGF levels secreted by the TGCs and the GVCs, an ELISA analysis was performed using the standardized Quantikine™ kit (R&D Systems, Lille, France) that

detects both secreted VEGF isoforms, i.e., $VEGF_{121}$ and $VEGF_{165}$. The sandwich ELISA was carried out according to the supplier's protocol, and optic densities were determined using an automated reader (Bio-Rad, Marnes-lacoquette, France). Concentrations of VEGF were determined by the Microman™ - reader software (Bio-Rad).

Cell migration and invasion assays

Migration in a modified Boyden chamber assay was performed essentially as previously described (14). For chemoinvasion, the filter was coated with a layer of Matrigel (0.5 mg/ml ; Becton Dickinson, Paris, France) as described (26). TGCs and GVCs were harvested by trypsinization, collected by centrifugation and resuspended in DMEM/F12 and EBM2 medium respectively with 0.5% BSA, washed 3 times with medium containing 0.5% BSA and resuspended in the same medium. At a concentration of 10^4 cells/ml, 100 µl of this suspension was added to the upper compartment (24-multiwell chemotaxis Boyden microchamber). The lower compartment of the chamber was filled with chemoattractant AM diluted in DMEM/F12 or EBM2. Where indicated, cells were incubated with an anti-AM antibody. After incubation at 37°C for 4 hours, cells that had not migrated from the top of the filter were scraped away with a cotton applicator. The filter was fixed for 30 minutes at room temperature with 3.7% paraformaldehyde, washed 2 times with PBS, and stained with hematoxylin. The number of cells that migrated to the lower surface of each membrane was counted. Each experimental point was analyzed in triplicate.

In vitro capillary tube formation in Matrigel

The morphogenesis assay on Matrigel was performed as previously described (27). GVCs maintained in EBM2 medium were washed twice with PBS, trypsinized and plated ($4x10^4$ cells) in wells coated with growth factor-depleted Matrigel (7mg/ml; Becton Dickinson, Paris, France) (28) in EBM2 supplemented with AM (50 nmol/l) or cotreatment with AM and anti-AM antibody (15µg/ml), (n=4 in duplicate). After 24 hours, the plates were photographed and the tube formation was qualitatively assessed.

Statistical analysis

Data are expressed as mean ± the standard error of the mean (SEM) from at least three independent experiments. Statistical analyses were performed by using the one-way ANOVA followed by Fisher's PLSD test (statview 512, Brain Power Inc, Calabasas, CA, USA).

Results

Glioblastomas primary cell culture

The aim of the present study was to identify the expression profile of AM and its receptors (CLR, RAMP2, RAMP3) in tumor glial cells (TGCs) and glioblastoma associated vascular cells (GVCs). Therefore we isolated GVCs from capillaries from individual human glioblastoma and further expanded primary cell cultures. Data obtained from cell cultures derived from six different glioblastomas specimens are presented. TGCs begin to adhere to the dish within 24 hours and then form a monolayer. On each glioblastoma primary culture, an immunolabelling assay was performed using anti-GFAP antibody, glial fibrillary acid protein (GFAP) is a specific protein for the cytoskeleton of astrocytes (Fig. 1a). The GFAP immunofluorescence demonstrates that 95% of the cells were GFAP positive. As immunofluorescence data showed a loss of GFAP staining with increasing number of passages, the cells were studied for the two first passages. Immunostaining for Tuj-1, a neuronal marker, was scarce (Fig. 1b), and a few cells (< 10%) were positive for SMA (Fig. 1c).

Development of vascular cells in culture

We performed characterization of isolated GVCs population by using endothelial specific antibodies against CD105, CD31 and CD146. After placing on collagen coated surface, GVCs grew in small isolated like colonies at different areas of the plate. After passages, cell shape changed to a more elongated form. Endothelial origin of the isolated cell population was detected by positive staining for CD105 (Fig. 1f). However cell culture and passaging may influence antigen expression in isolated GVCs. Stable antigen expression was found with CD105 whereas a short proportion of cells expressed CD31 and CD146 antigen (data not shown). To rule out any contamination with astrocytes, staining for GFAP was performed and no staining of was detected, indicating the absence of astrocytes in GVC's cultures (Fig. 1e). Positive

immunostaining for α-SMA, a marker for mature pericytes and myofibroblasts, was found at a range of 5% (Fig 1g).

AM and VEGF mRNA are differentially expressed in TGC and GVC.

Total RNA was prepared from primary TGCs and primary GVCs maintained in regular medium. To assess steady state levels of AM and VEGF transcripts we used a real time quantitative reverse transcription (QRT-PCR). Quantification of AM mRNA transcripts revealed the same pattern of expression in both cell types prepared from 7 tumors specimen (Fig 2). The mediane values of mRNA expression in glial and vascular compartment were 11.0 ua and 10.3 ua respectively (Fig 2). Quantification of VEGF mRNA transcripts showed a clear distinction between TGC and GVC with higher VEGF mRNA levels in glial cells compared to vascular cells with 6,85 and 1,5 mediane values respectively (Fig 2). VEGF mRNA expression showed 4.4 fold increase in TGC.

AM receptors (CLR, RAMP2, RAMP3) and VEGF receptors (flt1, KdR) are differentially expressed in TGC and GVC

The expression of CLR, RAMP2 and RAMP3 transcripts was analyzed by QRT-PCR reverse transcription from total RNA prepared for AM and VEGF analysis. Figure 3 shows higher expression of CLR in GVCs compared with TGCs. Quantification of CLR mRNA transcripts was 3.84 ua and 12.5 ua in TGC and GVC respectively.

A much higher difference in RAMP2 mRNA expression is observed between glial and vascular compartment. As shown in figure 3, RAMP2 mRNAs exhibited a 6,4 fold higher expression in GVCs (mediane level 60,9) as compared to TGCs (mediane levels 9,48). Inversely, RAMP3 is more expressed in TGCs compared with GVCs (median values 12,8 and 5,1 respectively).

Although there are differences at the expression level, these findings demonstrated that CLR, RAMP2 and RAMP3 are expressed in both cell types TGCs and GVCs issued from glioblastomas. VEGF receptors expression was also assessed, by VEGFR1 (flt1) and VEGFR2 (KDR) mRNAs transcripts, which were both preferentially expressed in glioma associated vascular cells (Fig 3).

The expression pattern of AM mRNA and AM receptors mRNAs was in parallel compared with the corresponding protein expression as shown by immunocytochemistry (Fig 4). AM staining was detected in glial and vascular cells (Fig. 4 a, f, left panels respectively) and was homogeneous and detectable mainly in their cytoplasm (Fig 4 a, f, right panels). The isolated TGC also exhibited a significant expression of the AM receptor as indicated by the presence of both CLR (Fig 4b), RAMP2 (Fig 4c) and RAMP3 (Fig 4d). As illustrated, GVCs also expressed CLR (Fig 4g), RAMP2 (Fig 4h) and RAMP3 (Fig 5i).

To confirm the presence of AM receptors proteins, cell extracts (40µg of proteins) from primary TGCs and GVCs maintained in regular medium, were subjected to Western blot analysis. Both, the glial cells (Fig 5, lanes 1-5) and the vascular cells (Fig 5, lanes 6-10) produced CLR as a distinct band of 48KDa and a multimer, presumably heterodimer, CLR/RAMP2, or CLR/RAMP3 at 73 KDa (Fig 5).

RAMP2 was seen as a monomer of 26KDa, and multimers, presumably homodimers at 48-50kDa (Fig 5). RAMP3 was seen as a monomer of 25KDa and 37KDa may represent some glycosylated monomer RAMP3 (Fig 5 lanes 4-5, lanes 8-10). Multimer RAMP3, presumably as homodimers, at 48KDa was also seen in all samples. These data demonstrate that CLR, RAMP2 and RAMP3 mRNAs and proteins are expressed in TGC as well as in GVC and may contribute to the function of AM.

TGCs and GVCs constitutively produce Adrenomedullin

The primary cells were cultured for 24 hours in regular medium. Subsequently culture supernatants were collected and analyzed for immunoreactive AM (ir-AM) by radioimmunoassay (RIA). Results show that GVCs produced ~ 3 fold higher amounts of AM compared with TGCs (Table 2). In one experiment (case 5) vascular cells secrete 7 fold times higher levels ir-AM compared with glial cells. Associated with AM, VEGF assessed by Elisa technique, accumulated in TGCs supernatants (Table 3).

TGCs and GVCs migration and invasion are induced by AM

To test whether AM could affect cell motility, TGCs and GVCs were incubated for 4 hours in a Boyden chamber with a range of AM concentration (1nmol/l-100nmol/l).

The number of cells that migrated to the lower surface of the transwell apparatus was increased dose-dependently in the AM treated glial cells (Fig 6A). A significant 98% increase in the number of migrated TGCs was detected with AM (1nmol/l). The optimal dose of AM for GVCs was 10 nmol/l (Fig 6B) with a 3 folds increase in the number of migrated cells. The invasive capacity of TGCs and GVCs in response to AM was investigated by measuring the invasion of a Matrigel layer in a Boyden chamber assay. Addition of AM at 10 nmol/l induced a 1.7 fold increase in TGCs invasion through Matrigel (Fig 6A). In GVCs, a 1,7 fold significative increase invasion was observed with AM at 10 nmol/l (Fig 6B). To test the autocrine hypothesis of AM effect, a polyclonal anti-AM Ab, developed and characterized in the laboratory, was examined for its effect on chemotaxis and chemoinvasion. Incubation of GVCS with anti-AM Ab (15 µg/ml) caused 40% inhibition of cell motility and invasion indicating that AM acts as an autocrine factor of GVC motility and invasion (Fig 6C). The decrease in the GVC migration treated with anti-AM Ab compared to control cells may reflect the action of endogenous AM secreted by endothelial cells. The same effect of anti-AM Ab was observed after incubation of TGCs with anti-AM Ab (data not shown).

AM induces *in vitro* morphologic differentiation of GVCs

To explore the ability of AM to induce capillary tube formation, we measured its ability to accelerate the formation of vascular structures in an *in vitro* Matrigel assay. After seeding on Matrigel, GVCs spread and aligned each other to form branching anastomosing tubes (Fig 7a). Addition of the synthetic AM peptide (100 nmol/l) resulted in the formation of a meshwork of increasing density (Fig 7b). As shown in Fig 7c, when the tests were performed in the presence of an AM neutralizing antibody, the morphogenetic effect of AM was no longer detectable. Next we tested the angiogenic activity of conditioned medium of TGCs (Fig 7d). Neutralization of AM by immunoprecipitation with an AM specific antibody reduced dramatically total tube length and vessel network (Fig 7e) indicating the specific contribution of AM secreted by TGCS to the arrangement and organization of VGCs into a meshwork of capillary-like tubular structures.

Discussion

Glioblastomas are the most malignant and common brain tumors. Malignant glioblastomas are highly aggressive angiogenesis dependent tumors characterized by rapidly dividing cells, invasion into normal brain and a high degree of vascularity. To provide the increasing blood supply as the tumor grows, cancer cells produce and secrete several proangiogenic growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). Adrenomedullin (AM), one such factor, belongs to a family of peptides that includes calcitonin (CT), α and β, calcitonin gene-related peptide (CGRP) and amylin. AM is a multifunctional peptide that was originally isolated from human pheochromocytoma, with properties ranging from including vasorelaxation to acting as a regulator of cellular growth (6). The role of AM in vascular physiology was demonstrated by an AM gene knockout mouse that showed embryonic lethality with severe abnormalities of the vasculature and subcuntaneous hemorrhage indicating the significance of AM signaling in vascular development (29, 30). AM is expressed in a variety of malignant tissues (10) and was shown to be mitogenic for human cancer cell lines including lung, breast, colon, prostate and brain lineages *in vitro* (7, 9, 10). AM is considered to be angiogenic in tumors (31, 32).

We demonstrated that the expression of AM mRNA was correlated to the tumor type and grade, with high expression in glioblastomas while a low expression was found in anaplastic astrocytomas and barely detectable levels in low grade astrocytomas and oligodendrogliomas (9). AM is a potent mitogen for glioblastoma cells and the neutralizing anti-AM antibody could be efficiently delivered *in vivo* and significantly suppress the growth of established glioblastoma xenografts. Furthermore the density of vessels with lumen was decreased in the antibody treated tumors, suggesting that AM might be involved in neovascularization and/or stabilization (9). In the current study we have used *in vitro* techniques to analyse the role of AM and AM receptors in tumor glial cells (TGCs) and vascular derived cells (GVCs) derived from six glioblastoma specimens obtained at surgery. We previously reported that AM is overexpressed in glioblastomas and stimulates cancer cells via an autocrine loop. We and others indicated that AM also promotes tumor growth by stimulation of angiogenesis through effects on endothelial cells in vitro and on cells within the tumor environment (9, 13, 14). Data presented here show for the first time that AM was expressed in both glioma and vascular cells as assessed by immunostaining of cultured cells. Immunohistochemical assays localized AM homogeneously mainly in the cytoplasm of the TGCs and GVCs.

Steady state levels of AM assessed by QRT-PCR revealed the same pattern of expression in both cell types prepared from tumor specimens. Herein, we showed the expression of CLR, RAMP2 and RAMP3 mRNAs. The Western blot analysis of cellular preparations demonstrate the presence of CLR, RAMP2 and RAMP3 proteins, suggesting that the heterodimers complexes (CLR/RAMP2, CLR/RAMP3) can occur in TGCs and GVCs and might be able to mediate the effects of AM. Furthermore we were able to detect AM in conditioned culture media from the GVCs which produced 3 fold higher amounts of AM compared with TGCs. Thus, AM is produced and secreted, and therefore able to interact with receptors including those of the cancer cells themselves as well as those on vascular cells in the tumor microenvironment. AM interacts with its receptors which are expressed by a broad range of cell types, including cancer cells, endothelial cells, mural cells, hematopoietic cells and myeloid cells. Recently, we demonstrated that AM is highly effective in recruiting endothelial cells and pericytes (13) as well as macrophage infiltration and myeloid precursor cells.

Endothelial secretion of AM is thought not only to coordinate vascular tone (33) but also to regulate fibroblast and vascular smooth muscle cell function (34, 35). AM is known to be expressed in endothelial cells of different organs. Active production of AM has been demonstrated in cultures vascular endothelial cells from mammals and constitutively secreted into the culture medium (36). More recently Tomoda et al (37) reported that endothelial cells, vascular smooth cells and fibroblasts generally secrete AM at high rates. To eliminate the possibility that contaminating glioma cells are responsible for AM production, immunohistochemical analysis were done, to confirm that GVCs were exclusively vascular cells. Data showed that endoglin (CD105) was exclusively expressed by vascular cells. CD105 is a proliferation associated cell membrane antigen of human vascular endothelial cells essential for angiogenesis (38). It has been shown overexpressed in blood vessels at the site of neovascularization (39).

To examine the possibility of an autocrine effect of AM on glioma cells, we determined the effect of exogeneous addition of AM *in vitro*. We demonstrated that AM significantly increased tumor glial cells migration and invasion through a matrigel layer. These data indicate that TGCs are able to respond to AM in ways that would be expected to the extremely high invasiveness that characterize glioblastomas (40). AM did not lead to a consistent growth stimulatory effect (data not shown). In contrast there

were significant effects of AM with respect to migration and invasiveness as it has been suggested in a subset of pancreatic cancer cells (41). Evidence is accumulating that AM possesses a significant proangiogenic effect. *In vitro* studies have shown a clear proangiogenic effect of AM in specialized vascular endothelial cells as Huvec (14, 42) or immortalized microvascular Endothelial cell lines (43).

In the present study, we provided evidence that treatment with AM induced proangiogenic phenotype in GVCs directly isolated from glioblastomas. Herein, AM stimulated migration, invasion and the formation of capillary tubes on matrigel by GVCs. GVCs migrate throughout the matrigel surface and aligne into a meshwork of capillary like tubular structure, when exposed to AM, the density and the complexity of the meshwork was increased. The activity of AM was specific because it was blocked by an AM antiserum in invasion, and in the capillary tube formation assay. Taken together, these findings and the presence of both AM and AM-R opens up for the possibility of AM being an autocrine angiogenic factor in GVCs isolated from tumor specimens.

In tumor cells, inflammation and hypoxia increase AM expression, and this elevated expression of AM is associated with tumor neovascularization. We tested the angiogenic activity of AM expressed in TGCs. When the vascular cells were exposed to conditioned medium of TGCs secreting AM and growth factors, we confirmed the tumor promoting effect of AM through direct angiogenic effect on GVCs. The neutralization of AM in conditioned medium, by immunoprecipitation with an AM specific antibody, reduced dramatically total tube length and vessel network. This significant inhibition of angiogenic activity obtained after immunoneutralization of AM strongly indicated that AM modulates angiogenesis via a paracrine mechanism. Several studies showed that tumor associated brain endothelial cells derived from glioma tissues secrete high amount of VEGF (44). In our present study, quantification of VEGF and VEGF receptor mRNA transcripts revealed a clear distinction between TGCs and GVCs with high levels of endogenous VEGF mRNA transcripts in TGCs and VEGF receptors (KDR/FLK1, FLT), mRNA transcripts mostly restricted to GVCs as described *in vivo* (2) consistent with the results obtained by Fernandez-Sauze et al. We confirmed that AM does not act through an upregulation of VEGF production, nor any regulator of angiogenesis ie, FGF, TGFb (data not shown) indicating that AM induced morphologic

differenciation does not require angiogenic factor.

Many basic investigations of endothelial cells have been performed on established endothelial cell lines; in addition, there has been no study on the direct effect of AM on tumor endothelial cells. However some evidence indicates that tumor endothelial cells differ from normal endothelial cells in terms of change in morphology, rate of cell proliferation, gene expression and drug sensitivity (45-47). Solid tumors are not just composed of malignant cells but are complex tumor-microenvironment interactions involving many cell types. AM might be involved in these interactions (13). The current study show that primary cultures of human TGCs and GVCs produce AM that acts as an autocrine/paracrine factor to induce cell migration and the multistep process of angiogenesis. Combined with the known importance of AM as an angiogenic factor, these data confirm the potency of AM and/or its receptor as new important targets for glioblastoma therapy as well as the treatment of tumors characterized by high levels of AM expression and active angiogenesis.

Figure 1: Immufluorescence of TGCs (a-c) and VGCs (d-f) for GFAP a marker of astrocytes (a), α-SMA a marker of mature pericytes (c,g), Tuj-1 a marker of neurons (b), CD105 a marker to detect endothelial cells in proliferative vessels (f) (20x). Nuclei were counterstained with DAPI (blue). Cells were incubated with a non immune serum as first layer (d,h).

Figure 2 and 3: Real-time quantitative RT-PCR analysis of AM, VEGF-A, CLR, RAMP2, RAMP3, and VEGF-R1, VEGF-R2 mRNA levels in TGCs and VGCs. Total RNA DNA-free from primary cultured cells was reverse transcribed to cDNA and subjected to real-time quantitative RT-PCR for the estimation of relative AM, VEGF-A, CLR, RAMP2, RAMP3, VEGF-R1, VEGF-R2 mRNA to 18S rRNA ration as described in "Materials and Methods", and presented as arbitrary units. Bars represent the mean ± SEM of the mRNA expression values for each parameter as indicated.

Figure 4: Immunocytochemistry for AM, CRL, RAMP2 and RAMP3 expression (n=4) in TGCs (a-e) and VGCs (f-j) were performed as described in the Methods section. Magnification: x20 to the left panels and x60 to the right panels.

Figure 5: Expression of CLR, RAMP2, and RAMP3 in TGCs and VGCs in 5 individual gliblastomas specimen. Aliquots of protein extracts (40µg) of TGCs (lane 1-5) and of GVCs (lane 6-10) were separated on 12% SDS-PAGE, transferred to Hybond-C membrane, and immunoblotted using antibodies direct against CLR, RAMP2 and RAMP3 peptides as described previously (Fernandez-Sauze et al., 2004). To assess the amount of proteins loaded for each sample, immunoblot analysis was performed with anti-β-actin.

Figure 6: Effect of AM on TGCs and GVCs migration and invasion. TGCs (A : $1x10^4$ cells) or GVCs (B,C : 1.10^4 cells) treated with anti-AM or IgG control were placed in the upper compartment of a 24-well Boyden chamber. AM (1,10, 100nmol/l) was added in lower wells. Cells migrating through the filter in absence or presence of Matrigel were counted after 4 hours. Data are expressed as the number of migrated cells in 10 high-power fields, and the values represent the mean ± SEM of 4 independent experiments each performed in triplicate.

Figure 7: Morphogenetic activity of AM phase contrast micrographs illustrating the arrangement of VGCs ($4x10^4$ cells/wells) seeded in to Matrigel-precoated well for 18 hours in absence (a) or in presence of 100 nmol/l AM (b), 100 nmol/l AM + anti-AM antibody (15µg/ml) (c), or with conditioned medium of TGCs before (d) and after AM depletion by immunoprecipitation (e). Original magnification x40.

Table 2 : Production of IR-AM by 24h cultured TGCs and GVCs of human glioblastoma. Results are expressed in $pg/10^6$ cells. Values are mean ± sd.

Table 3 : Production of IR-AM and VEGF by 24h cultured TGCs of human glioblastomas. Values are mean ± sd.

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Fig. 1

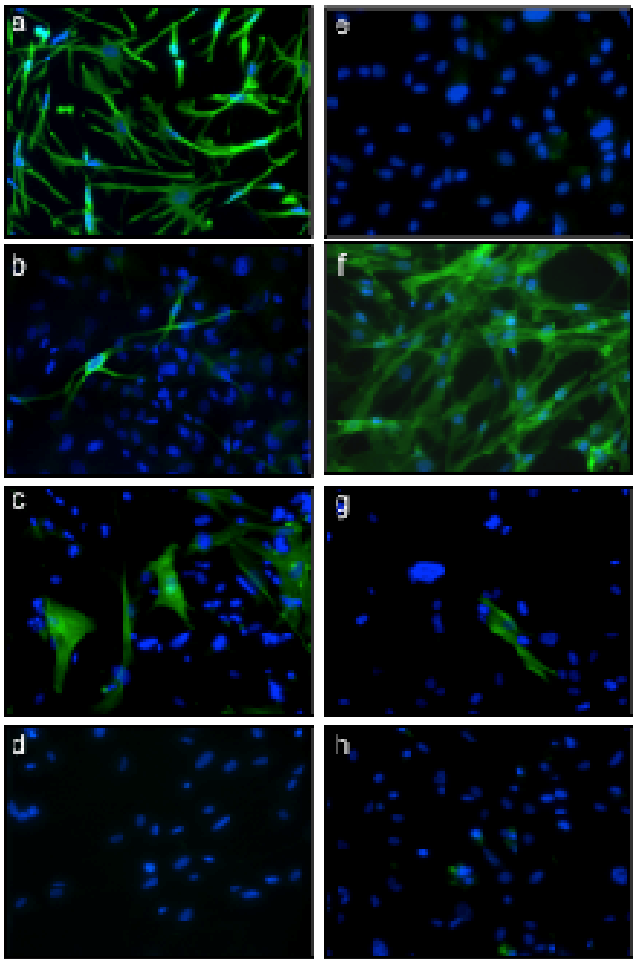


Fig. 2

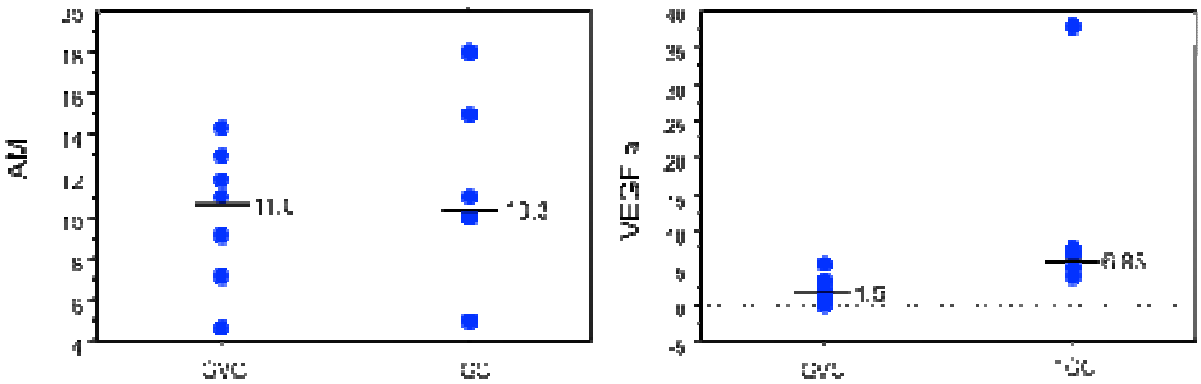


Fig. 3

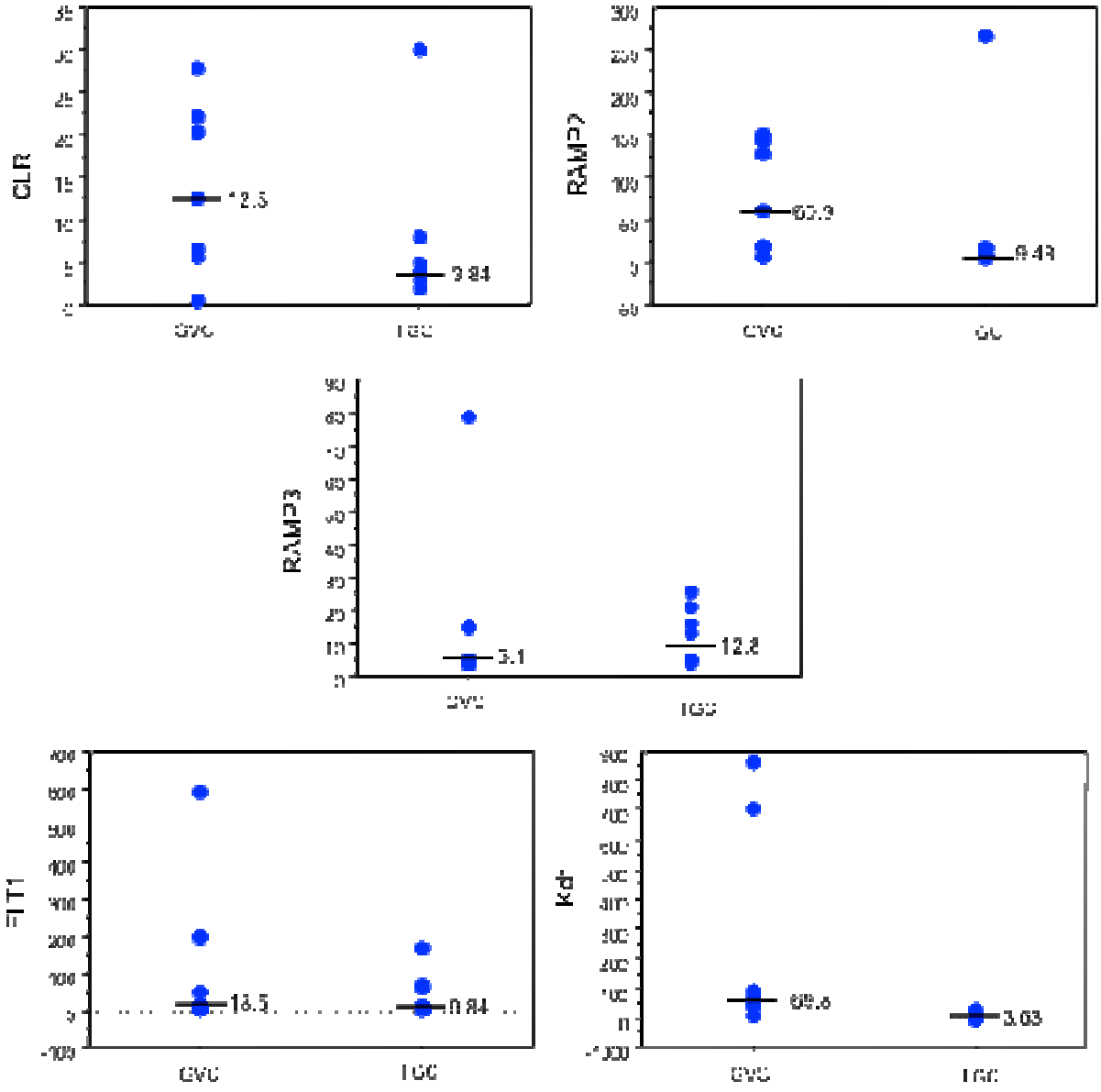
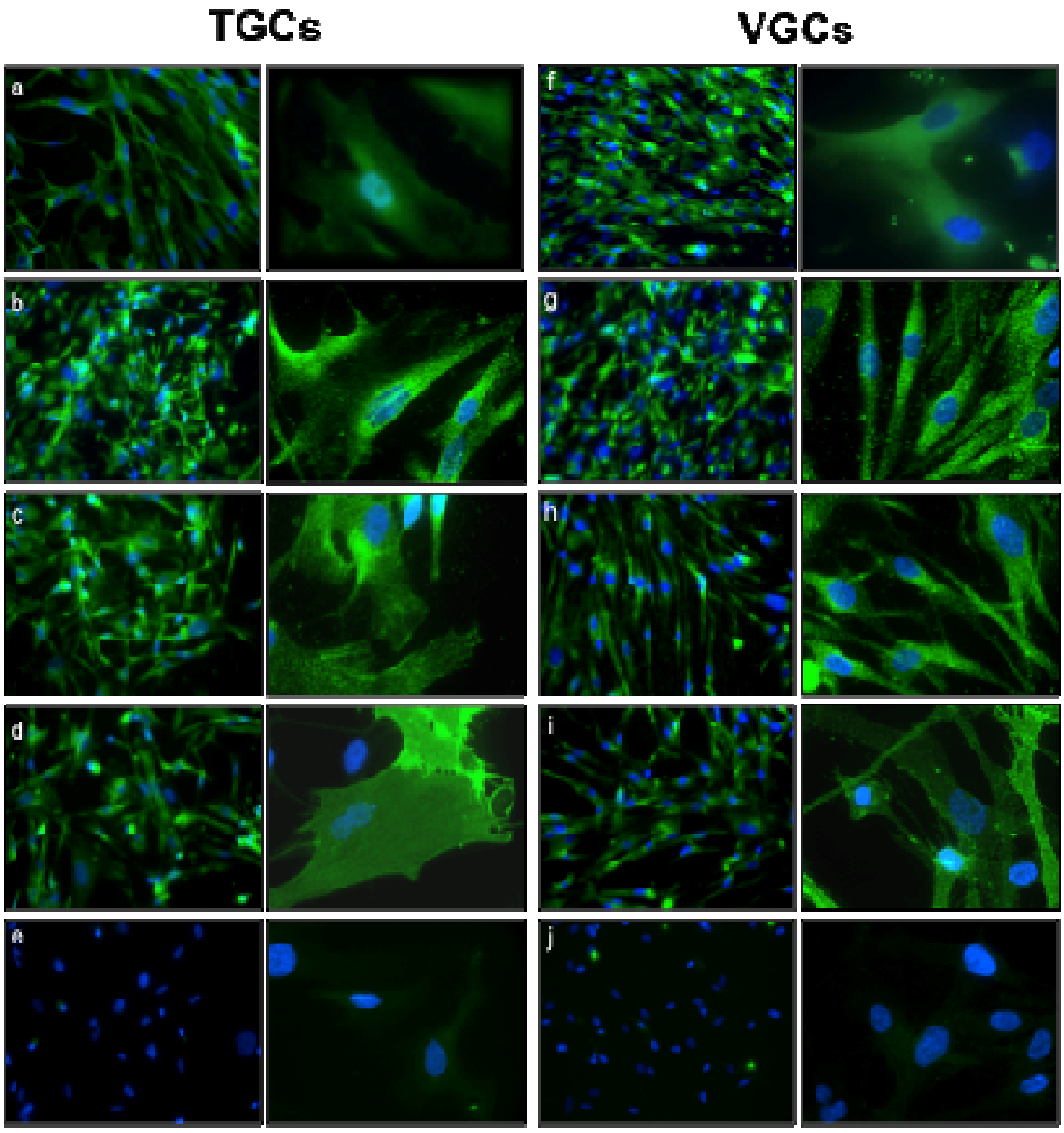


Fig. 4



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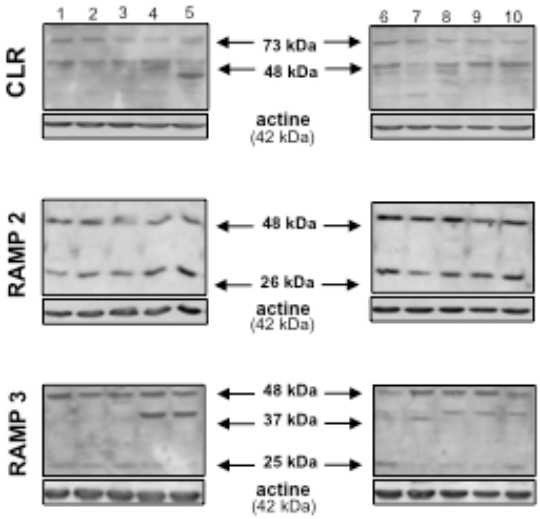


	Tumoral glial cells (TGCs)	Tumoral vascular cells (GVCs)
GBM1	8,80 = 3.10	25.86 ± 2.39
GBM2	6,52 = 1.95	15.17 ± 3.52
GBM3	7,65 = 2.65	21,77 = 4.9
GBM4	13.36 ± 4,75	33.41 ± 6.41
GBM5	8,21 = 3.83	57.31 ± 12.18
GBM6	4,83 = 0.32	18,36 = 4.7

Table 2 : Production of IR-AM by 24h cultured TGCs and GVCs of human glioblastomas. Results are expressed in pg/10^6 cells. Values are mean ± sd.

Fig. 6

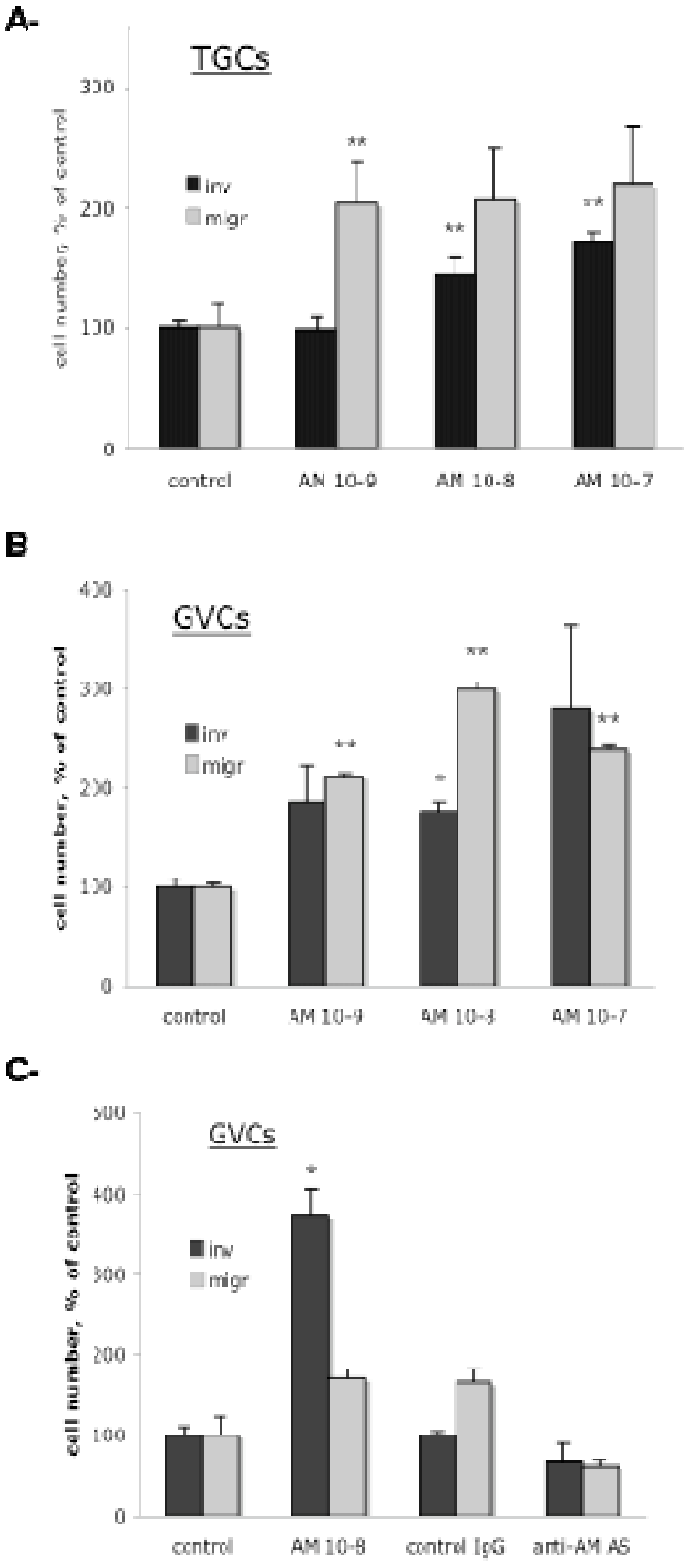
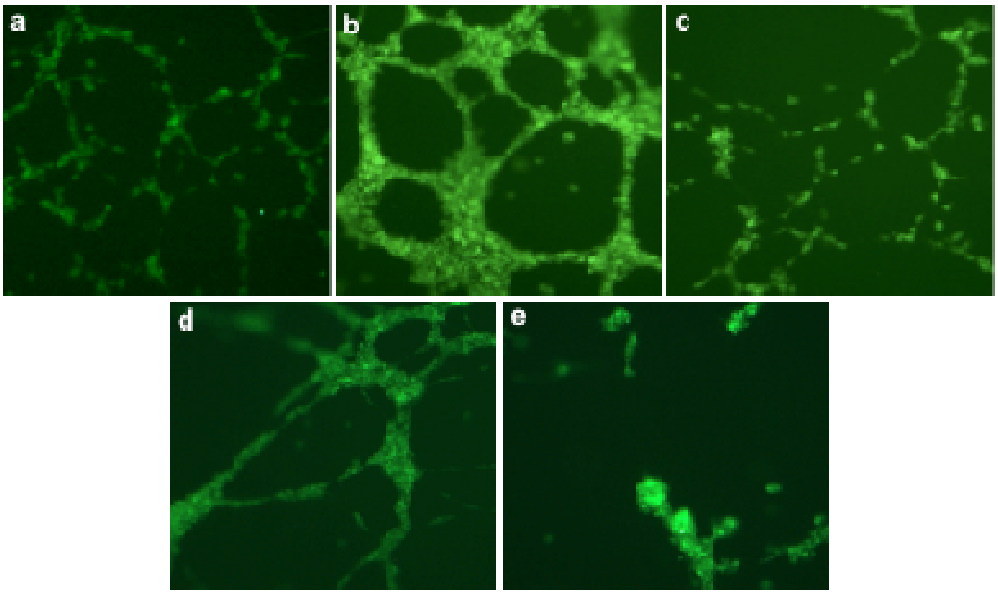

A-
TGCs
cell number, % of control
300
200
100
0
inv
migr
**
**
**
control
AM 10-9
AM 10-8
AM 10-7
B
GVCs
cell number, % of control
400
300
200
100
0
inv
migr
**
**
*
**
control
AM 10-9
AM 10-8
AM 10-7
C-
GVCs
cell number, % of control
500
400
300
200
100
0
inv
migr
*
control
AM 10-8
control IgG
anti-AM AS

Fig. 7



	AM (pg / µg protein/ 24h)	VEGF (pg / µg protein/ 24h)
GBM1	14.04 ± 7,01	302.10 ± 66,17
GBM2	4,29 = 1.68	371.35 ± 80,98
GBM3	1,80 = 0.30	446,11 ± 64.26
GBM4	13.37 ± 5,67	321.4 ± 32,9
GBM5	19.61 ± 7,15	176.51 ± 84,17
GBM6	15.12 ± 4,67	252.34 ± 41,63

Table 3 : Production of IR-AM and VEGF by 24h cultured TGCs of human glioblastomas. Values are mean ± sd.

Table 1- Sequences of forward and reverse primersand probes used to quantify human AM, VEGF, CLR, RAMP2, RAMP4 mRNA, and 18srRNA. Corresponing Q-PCR conditions are also given

Gene	Primers, fluorescence probes, PCRconditions
AM	Forward primer 5'TGCCCAGACCCTTATTCGG-3' Reverse primer 5'-AGTTGTTCATGCTCTGGCGG-3' Probe FAM-ACATGAAGGGTGCCTCTCGAAGCCC-TAMRA (95C°fo r 15' ; 45 cycles at 94C°for 15'', 67C°for 20'')
VEGF	Forward primer 5'-AGGAGGAGGGCAGAATCATCA-3' Reverse primer 5'-AGGGTCTCGATTGGATGGC-3' Probe FAM-TGAAGTTCATGGATGTCTATCAGGCGCAGCT-TAMRA (95C°for 15' ; 45 cycles at 94C°for 15'', 66C°fo r 15'')
CLR	Forward primer 5'-TGGCTTAATGATGGAGAAAAAGTG-3' Reverse primer 5'-TCAGGACTCTCTTCTAATTCTGCTG-3' Probe FAM-CCTGTATTTTCTGGTTCTCTTGCCTTTTTTTTATGA-TAMRA (94C°fo r 15' ; 40 cycles at 94C°for 20'', 60C°for 20'')
RAMP2	Forward primer 5'-GACGGTGAAGAACTATGAGACAGC-3' Reverse primer 5'-GCTATAAGGCTGCTAATCATGG -3' Probe FAM-TGGATCCTATCGAAAAGGATTGGTGCG-TAMRA (95C°for 15' ; 40 cycles at 94C°for 20'', 67C°for 30'')
RAMP3	Forward primer 5'-TCTGGAAGTGGTGCAACCTGT-3' Reverse primer 5'-GATGCCGGTGATGAAGCC-3' Probe FAM-AGATGGAGGCCAATGTCGTGGGCT-TAMRA (95C°for 15' ; 40 cycles at 94C°for 20'', 67C°for 30'')
18s	Forward primer 5'-CTACCACATCCAAGGAAGGCA-3' Reverse primer 5'-TTTTTCGTCACTACCTCCCCG-3' ProbeFAM-CGCGCAAATTACCCACTCCCGAC-TAMRA (95C°for 15' ; 40 cycles at 94C°for 15'', 67C°for 15'')

FAM, 5-carboxyfluorescein, TAMRA 5-carboxytetramethylrhodamin

DISCUSSION ET PERSPECTIVES

I.DISCUSSION

1. Implication de l'AM dans certaines étapes clés de l'angiogenèse tumorale

L'AM est d'avantage secrétée par la composante microvasculaire des glioblastomes

L'analyse de l'expression de l'AM permet de démontrer une expression plus élevée de l'AM dans les glioblastomes comparés aux astrocytomes pilocytiques. Cette expression peut être corrélée avec la proximité des zones de nécrose où l'oxygène est minimal. L'AM est induite spécifiquement en réponse à l'hypoxie.

L'analyse de l'expression des ARNm par RT-PCR et des protéines par immunohistochimie, Western-blot et dosage radio-immunologique, permet d'affirmer que la composante gliale tumorale et la vascularisation des glioblastomes expriment l'AM et ses récepteurs CLR, RAMP2 et RAMP3, suggérant une action autocrine et paracrine sur ces cellules et une interaction entre elles via l'AM.

Effet chimioattractant de l'AM

Après le déclenchement de l'angiogenèse, les cellules endothéliales microvasculaires des vaisseaux en formation sont activées. Elles prolifèrent, migrent vers les sites en hypoxie et s'organisent pour former les vaisseaux néoformés.

Une hétérogénéité des résultats sur l'effet de l'AM dans la prolifération cellulaire nous a mené à penser que là n'était pas le rôle principal de l'AM. En revanche, l'action chimioattractante de l'AM dans la migration et l'invasion des cellules microvasculaires est bien plus évidente avec une invasion doublant et une migration triplant sous l'effet chimioattractant de l'AM. Ce résultat nous permet de suggérer que l'AM sécrétée par les cellules gliales tumorales peut par chimioattraction induire la migration et l'invasion des cellules endothéliales des vaisseaux en formation afin de les attirer pour venir en

aide aux cellules souffrant d'hypoxie. Tel le VEGF surexprimé par les cellules tumorales au niveau des palissades nécrotiques, les cellules gliales tumorales en hypoxie secréteraient de l'AM afin d'attirer de nouveaux vaisseaux qui pallieraient au manque d'oxygène et de nutriments.

D'autre part, la composante microvasculaire étant majoritairement composée de cellules endothéliales, la forte expression d'AM par ces cellules pourrait activer de façon autocrine/paracrine leur propre migration et invasion mais également celle des péricytes de façon paracrine. Dans ce cas l'AM participerait à la phase de maturation des vaisseaux néoformés. Il s'avérerait très intéressant de caractériser le système AM/AMR au sein des cellules endothéliales et des péricytes provenant de la composante microvasculaire des glioblastomes afin d 'étudier cette hypothèse et le rôle de l'AM dans les interactions entre ces deux types cellulaires.

Effet de l'AM dans l'organisation en pseudo-capillaire

Les expériences de formation en pseudo-capillaire des cellules microvasculaires issues de glioblastomes sur Matrigel permettent de mettre en évidence l'importance de l'AM dans ce phénomène. Mais de façon plus intéressante, cette expérience montre que l'AM sécrétée par les cellules tumorales participe à la formation des vaisseaux. Ce qui est cohérant avec l'hypothèse évoquée selon laquelle l'AM sécrétée par la composante tumorale activerait l'angiogenèse tumorale.

2. L'AM stimule par chimioattraction la migration et l'invasion des cellules gliales du glioblastome

Dans notre étude, nous démontrons que l'AM active de façon dose dépendante la migration et l'invasion des cellules gliales tumorales provenant des glioblastomes.

L'invasivité des cellules tumorales des glioblastomes étant l'un des principaux critères de malignité et l'une des causes de récidive qui rendent ces tumeurs fatales, ce résultat est important. Ces résultats ont d'autant plus d'importance que l'une des problématiques des thérapies anti-angiogéniques est d 'induire l'invasion par la tumeur du tissu sain voisin épargné par les traitements. La tumeur étant attaquée d'un côté pour se déplacer de l'autre.

II.PERSPECTIVES

1. Vers un essai Clinique

Plus de 30% des essais cliniques sont actuellement consacrés aux anticorps monoclonaux. Les avantages de ces anticorps par rapport aux anticorps polyclonaux trouvés dans un sérum sont importants. Les hybridomes sont gardés pendant des années en culture *in vitro* sans modification de l'anticorps qu'ils produisent. De plus, ces cellules peuvent être congelées. Une source illimitée de cellules produit alors toujours le même anticorps ayant la même affinité et les mêmes propriétés physicochimiques. Le laboratoire envisage à court terme le développement d'anticorps monoclonaux. Or la taille du peptide chimère est de 78 acides aminés. Pour cela, il est fondamental de diminuer la longueur de la séquence peptidique. Cela nécessite la connaissance des épitopes reconnus par les anticorps dans les différentes séquences impliquées dans la chimère. La synthèse chimique des différents peptides du coté N-ter et C-ter est en cours. Ce travail nous permettra de déterminer les différents épitopes impliqués dans la génération des anticorps et de mettre en place la synthése d'une séquence chimérique raccourcie contre laquelle des anticorps monoclonaux seront développés. De tels anticorps pourraient par la suite présenter le début d'une thérapeutique ciblant le

système AM/AMR et représenteraient un gain de temps pour faire accepter ce système en thérapeutique.

2. **Comprendre les mécanismes sous-jacents à l'inhibition de l'angiogenèse et de la croissance tumorale lors du ciblage du système AM/AMR**

Nos travaux ont permis de montrer que le blocage du système AM/AMR était responsable d'une inhibition de l'angiogenèse et de la croissance tumorale in vivo. Cependant les voies cellulaires et moléculaires par lesquelles le blocage du système AM/AMR entraine une suppression de l'angiogenèse et de la croissance tumorale restent mal définies. Des travaux en cours étudient les interactions entre cellules endothéliales et péricytes. Les résultats préliminaires tendent à montrer que l'AM joue un rôle majeur dans la maturation et la stabilité du réseau vasculaire tumoral via un complexe endothélial spécifique VE-cadherin/beta-catenin. La perturbation du signal VE-cadherin/beta-catenin/Akt par un blocage du système AM/AMR serait à l'origine de la déstructuration et de la réduction du réseau vasculaire et de l'inhibition de la croissance tumorale.

CONCLUSION

Les glioblastomes sont des tumeurs fatales du fait de leur agressivité et du manque de traitements efficaces. La prolifération accrue, le caractère invasif et la résistance à la mort cellulaire du compartiment tumoral des glioblastomes leur confèrent une croissance rapide et une invasion du parenchyme cérébral environnant, à l'origine de leur systématique récidive. De plus le processus d'angiogenèse au sein de ces tumeurs participe activement au mauvais pronostic en développant une forte vascularisation qui favorise leur croissance.

Exprimée par la composante tumorale en hypoxie mais également par la composante vasculaire, l'AM participe de façon autocrine et paracrine au développement des glioblastomes en favorisant d'une part la croissance des cellules tumorales et d'autre part l'angiogenèse tumorale.

Il a été démontré au sein de notre équipe que des anticorps polyclonaux anti-AM inhibent la prolifération des cellules gliales tumorales *in vitro* ainsi que la croissance tumorale *in vivo*. (Ouafik et al., 2002). Il a été montré que des anticorps polyclonaux dirigés contre les récepteurs de l'AM inhibent *in vitro* la croissance, la migration et la formation de pseudo-capillaires des cellules endothéliales, suggérant une neutralisation par ces anticorps de certaines étapes de l'angiogenèse. De même, il a été montré in vivo que ces anticorps inhibent la croissance tumorale en supprimant l'angiogenèse et la croissance des cellules tumorales suggérant ainsi que les récepteurs de l'AM constitueraient une bonne cible thérapeutique.

Des anticorps capables de reconnaître et neutraliser à la fois l'AM, les CLR, RAMP2 et RAMP3 agissant de la même manière sur la croissance tumorale et l'angiogenèse représenteraient un bénéfice thérapeutique majeur. Le laboratoire est en train de développer des anticorps dirigés contre un peptide chimérique constitué de l'enchaînement de séquences peptidiques des protéines CLR, RAMP2, RAMP3 et du

peptide AM. Les tests effectués nous permettent d'affirmer une efficacité sur le système AM/AMR. Le traitement par ces anticorps diminue la croissance *in vitro* et *in vivo* des cellules tumorales et glioblastomes U87 ainsi que leurs migration et invasion. De plus, le traitement par ces anticorps augmente la perméabilité du modèle de cellules endothéliales microvasculaires HEMCs. Ces résultats très encourageants nous permettent pour le moment de valider la faisabilité du concept d'anticorps développés à partir d'un peptide chimérique pour neutraliser le système AM/AMR dans le but d'envisager dans le futur une application thérapeutique.

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Les glioblastomes sont des tumeurs fatales du fait de leur agressivité et du manque de traitements efficaces. La prolifération accrue, le caractère invasif et la résistance à la mort cellulaire leur confèrent une croissance rapide et une invasion du parenchyme cérébral environnant, à l'origine de leur systématique récidive. Exprimée par la composante tumorale en hypoxie mais également par la composante vasculaire, l'AM participe de façon autocrine et paracrine au développement des glioblastomes en favorisant la croissance des cellules tumorales et l'angiogenèse tumorale.

Il a été montré que des anticorps polyclonaux dirigés contre les récepteurs de l'AM inhibent *in vitro* la croissance, la migration et la formation de pseudo-capillaires des cellules endothéliales, suggérant une neutralisation par ces anticorps de certaines étapes de l'angiogenèse. De même, il a été montré *in vivo* que ces anticorps inhibent la croissance tumorale en supprimant l'angiogenèse et la croissance des cellules tumorales suggérant ainsi que les récepteurs de l'AM constitueraient une bonne cible thérapeutique. Des anticorps capables de reconnaître et neutraliser à la fois l'AM, les CLR, RAMP2 et RAMP3 agissant de la même manière sur la croissance tumorale et l'angiogenèse représenteraient un bénéfice thérapeutique majeur. Des anticorps dirigés contre un peptide chimérique constitué de l'enchainement de séquences peptidiques des protéines CLR, RAMP2, RAMP3 et du peptide AM sont en cours. Le traitement par ces anticorps diminue la croissance des cellules tumorales ainsi que leurs migration et invasion. Ces résultats très encourageants nous permettent pour le moment de valider la faisabilité du concept d'anticorps développés à partir d'un peptide chimérique pour neutraliser le système AM/AMR dans le but d'envisager dans le futur une application thérapeutique.