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Introduction

PRIMARY BRAIN TUMORS

Primary brain tumours consist of a diverse group of neoplasms that are derived from various different cell lineages of the central nervous system (CNS).
About 80% of malignant primary brain tumors are known collectively as gliomas.
The most widely used scheme for classification and grading of gliomas is the one stated by the World Health Organization (WHO). Gliomas are classified according to their hypothesized line of differentiation, that is, whether they display features of astrocytic, oligodendroglial, or ependymal cells. They are then graded on a scale of I to IV according to their degree of malignancy. Unlike other solid tumors, gliomas very rarely metastasize outside the CNS, and thus tumor grade is the primary determinant of clinical outcome. Grade I tumors are biologically benign and can be surgically cured if deemed resectable at the time of diagnosis; grade II tumors are low-grade malignancies that may follow long clinical courses but are not cureable by surgery; grade III tumors are malignant and lead to death within a few years; grade IV tumors are highly malignant, usually recalcitrant to chemotherapy, and lethal within 9–12 months. Seventy percent of grade II gliomas transform into grade III and IV tumors within 5–10 years of diagnosis and then behave clinically like the higher-grade tumors.
There are 3 main types of glioma: astrocytoma, ependymoma and oligodendroglioma and there are several glial cell types from which gliomas form:

- Astrocytomas are primary brain tumors derived from astrocytes and consist of about 60% of all malignant primary brain tumors. Astrocytoma tumor types by grade include:

  Grade I. Pilocytic astrocytoma is one of the most common types of glioma in children;

  Grade II. Diffuse astrocytoma (also called low-grade astrocytoma) typically occurs in men and women aged 20–60;

  Grade III. Anaplastic astrocytoma typically occurs in adults aged 30-60, and is more common among men than women;

Grade IV. Glioblastoma multiforme (GBM) accounts for about 50% of all astrocytomas. These highly malignant aggressive tumors are most common in older adults (50s-70s), particularly men. Only about 10% of childhood brain tumors are glioblastomas.

- Oligodendrogliomas develop from oligodendrocyte glial cells, which form the protective coatings around nerve cells. Oligodendrogliomas are classified as either low-grade (grade II) or anaplastic (grade III). Oligodendrogliomas usually occur in younger and middle-aged adults.
- Ependymomas are derived from ependymal cells, which line the ventricles (fluid-filled cavities) in the lower part of the brain and the central canal of the spinal cord. They are one of the most common types of brain tumor in children. They can also occur in adults in their 40s and 50s. Ependymomas are divided into four categories: Myxopapillar ependymomas (grade I), subependymomas (grade I), ependymomas (grade II), and anaplastic ependymomas (grades III and IV).
- Mixed gliomas contain a mixture of malignant gliomas. About half of these tumors contain cancerous oligodendrocytes and astrocytes.

Gliomas may also contain cancer cells derived from brain cells other than glial cells.

Work in both animal models and primary gliomas suggests the more likely possibility that malignant gliomas arise from neural progenitor cells. Neuroectodermal stem cells that reside in adult mammalian brains have proliferative potential, are migratory, and can pursue diverse paths of differentiation; all these features are intrinsic to glioma cells and likely characteristics of neoplastic cells of origin. Thus, there is a large possibility that the endogenous adult neuroectodermal stem cells are the cells of origin for primary brain tumors. Differential targeting of oncogenic events to specific cell types can be accomplished by restricting the expression of a viral receptor to either progenitor cells or maturing astrocytes, followed by the introduction of oncogenes in specific viral vectors (Holland et al, 2000). Furthermore, malignant gliomas likely contain tumor stem cells, a relatively primitive population responsible for populating and repopulating the tumors as they develop and progress (Galli et al, 2004; Singh et al, 2004; Singh et al, 2003); such tumor stem cells may be transformed variants of normal neural progenitor cells.

Neoplastic transformation

The earliest events in glial neoplastic transformation remain unclear. Nonetheless, the genetic events most common in grade II gliomas differ between the two major types, astrocytoma and oligodendroglioma.

In grade II astrocytomas, inactivating *TP53* mutations are found in more than 50% of tumors (Okamoto et al, 2004). Mouse models have also demonstrated that p53 inactivation contributes to astrocytoma tumorigenesis (Reilly et al, 2000). Thus, the abrogation of astrocytic p53 function appears to facilitate some events integral to neoplastic transformation, setting the stage for further malignant progression. In addition, in higher-grade astrocytomas, the p53 pathway may be deregulated by alterations of other components, including amplification of *MDM2* or *MDM4* and 9p deletions that result in loss of the ARF product of the *CDKN2A* gene (Ichimura et al, 1996; Ichimura et al, 2000).

Allelic loss in oligodendrogliomas occur preferentially on chromosomes 1p and 19q, affecting 40%–80% of these tumor types (Reifenberger and Louis 2003). These losses are closely associated; oligodendroglial tumors with 1p loss usually also have 19q loss, which suggests that the two putative tumor suppressor genes located in these chromosomal segments may be involved in biologically distinct pathways that are linked in some functional manner.

An understanding of glioma phenotype must also take into account the observations that astrocytic tumors are associated with *TP53* mutations, and oligodendrogliomas with 1p and 19q loss. It is possible that astrocytomas and oligodendrogliomas arise from the same cells of origin, but different genetic events drive differentiation along different lines. Alternatively, astrocytomas and oligodendrogliomas may arise from different cells of origin that are oncogenically permissive only for particular events. Note that oligodendroglial tumors with 1p and 19q loss preferentially affect particular areas of the brain, raising the possibility that oncogenesis in specific precursor cell populations in different brain regions must proceed along distinct genetic pathways to reach the common phenotypic endpoint of oligodendroglioma (Zlatescu et al, 2001) (Figure 1).

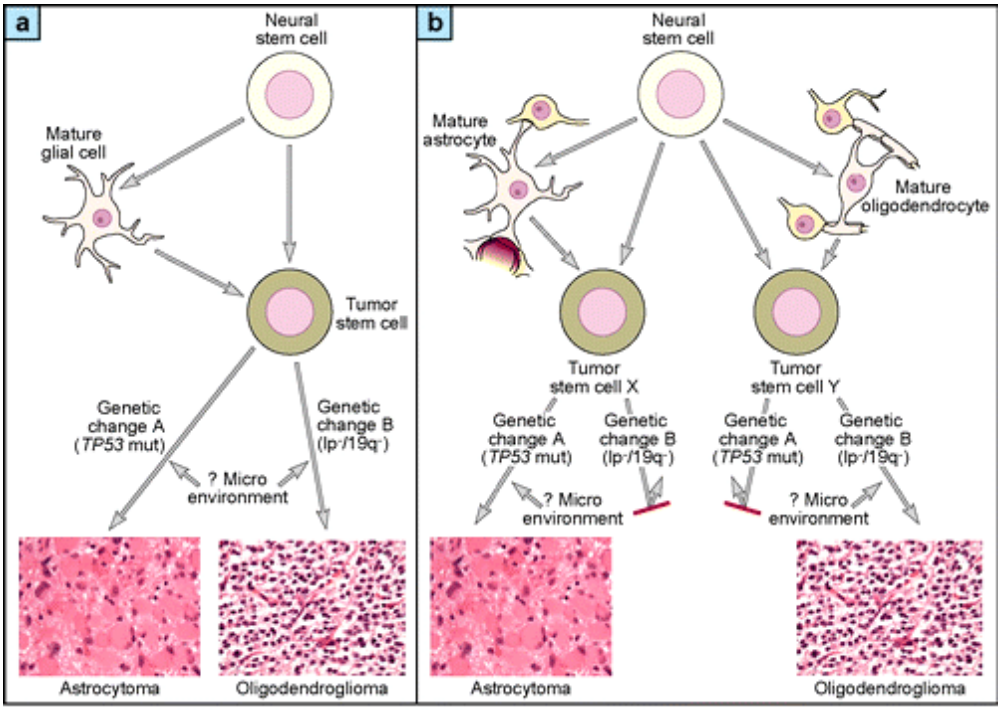

Figure 1: Stem cells and tumor differentiation. (a) A single type of tumor stem cell can arise from a neural stem cell or from a mature glial cell. The end phenotype of astrocytoma or oligodendroglioma depends upon the activation of particular cellular pathways, either as a result of undergoing one set of genetic changes (e.g., TP53 mutation) or another (e.g., 1p and 19q loss), possibly influenced by the tumor micro-environment. (b) Possible different types of tumor stem cells for various tumor types. In this model, one tumor stem cell (X) is permissive for neoplastic transformation only in the setting of particular genetic changes (e.g., TP53 mutation), perhaps partially as a result of micro-environmental factors; other genetic changes (e.g., 1p and 19q loss) are lethal, hence the restriction to an astrocytic phenotype. Another stem cell (Y), perhaps influenced by micro-environmental factors, undergoes tumorigenesis only in the setting of other genetic changes (e.g., 1p and 19q loss), yielding an oligodendroglial tumor (Louis, 2006).

Invasion

Gliomas have a remarkable tendency to infiltrate the surrounding brain. These invasive abilities are often apparent in low-grade as well as highgrade tumors, implying that the invasive phenotype is acquired early in tumorigenesis.

Glioma invasion must be viewed as a combination of the ability to migrate and the ability to modulate the extracellular space. Invasion of glioma cells into the brain most likely reflects a dynamic interplay between cell-cell adhesion, remodeling of the extracellular matrix, and cell motility (Cavenee et al, 2000). But invasion into brain parenchyma is biologically distinct from invasion of

carcinomas into adjacent tissues in that the extracellular matrix of the brain is distinct from the extracellular matrix of most organs.

The neural ECM has a unique molecular composition, containing very small amounts of collagens, laminin-1 and fibronectin (Ruoslahti, 1996). This matrix is primarily composed of a hyaluronic acid (HA) scaffold, which behaves as a large molecular sieving mesh that influences cell division and motility. This scaffold has associated glycoproteins and proteoglycans (PGs) which regulate its molecular properties and spatial localization, and are crucial in determining the major structural features of the neural ECM. The predominant group of HA-binding proteins in the neural ECM is the lectican family of chondroitin sulfate proteoglycans (CSPGs), which provide a structural and functional link between the cell membrane and the HA-based matrix scaffold (Yamaguchi, 2000). These are the most abundant CSPGs in the adult CNS and have a central role as matrix 'organizers' owing to their pericellular localization and multiple interactions with ECM and cell-surface molecules.

The lectican family is composed of four members in mammals: two of them (aggrecan and versican) are widely distributed, whereas the other two (neurocan and brain enriched hyaluronan-binding protein, BEHAB, also known as brevican or B/b) are mainly restricted to the CNS. All lecticans are secreted CSPGs with a structure that consists of a major globular domain at each end, joined by a stretched domain that carries the CS chains (Oohira et al, 2000). The binding of the terminal domains to HA and cell-surface receptors suggests an anchoring function for the lecticans that might limit cell or neurite motility. In addition, the CS chains are negatively charged at physiological pH, which might be a source of electrostatic repulsion underlying the role of the lecticans as strong negative signals for navigating axons (Morgenstern et al, 2002) or motile cells (Bandtlow and Zimmermann, 2000). However, the lecticans exhibit a complex heterogeneity of structures, expression patterns and interactions that are suggestive of other functions in addition to their major role as structural components and chemorepulsants in the CNS. The lecticans also exhibit important differences in their spatiotemporal expression in the CNS. During development, the expression of versican and neurocan peaks during early development and decreases markedly shortly after birth, whereas aggrecan and brevican are expressed at low levels during early ontogeny but increase markedly in the adult CNS (Viapiano et al, 2003; Maeda et al, 2006). Several CSPGs, including the lecticans versican and B/b, are overexpressed in gliomas and promote tumor growth, vascularization and invasiveness (Nutt et al, 2001; Paulus et al, 1996). Because these CSPGs inhibit

cell and process movement in the adult CNS, their motogenic effect in gliomas is surprising and seems unrelated to their inhibitory properties in normal tissue. However, given the changes observed for other ECM and cell-surface molecules in gliomas, it is possible that the CSPGs exhibit novel interactions with tumor-expressed ligands. Additionally, the CSPGs themselves might be differentially modified in gliomas because of tumor-specific post-translational processing. Thus, changes in the expression and molecular interactions of the lecticans might underlie novel functions of these molecules that would enhance, instead of limiting, the motogenic strategy of tumor cells.

Versican is expressed in the parenchyma and vasculature of primary brain tumors (Paulus et al, 1996). In vitro studies have shown that the N-terminal globular domain of versican can directly promote glioma-cell motility, whereas the C-terminal domain promotes cell adhesion (Ang et al, 1999; Wu et al, 2002). The specific molecular mechanisms underlying these effects are still mostly speculative, but it is known that the C-terminal domain of versican can activate EFG receptor signaling and β-1 integrin engagement, the latter causing phosphorylation of focal adhesion kinase. Additionally, this C-terminal domain promotes endothelial-cell proliferation and glioma vascularization (Zheng et al, 2004). Although most of these results have been obtained with engineered C-terminal fragments of versican, actual protein fragments corresponding to this domain are found in astrocytomas and in the conditioned medium of glioblastoma cell lines, and they are likely to be produced by matrix metalloprotease (MMP) degradation of the full-length protein.

Investigations into astrocytoma invasion have highlighted the complex nature of cell-cell and cell-extracellular matrix interactions (Rao, 2003). Proteases are elaborated by glioma cells (including cysteine, serine, and metalloproteinases) and appear to play a significant role. These selective proteases degrade the extracellular environment in order to facilitate migration, but also likely remodel the environment in a manner that facilitates tumor cell growth. A number of proteases have been implicated, but most studies have focused on matrix metalloproteinases (MMPs). MMPs are the largest group of ECM-degrading enzymes; so far 22 members have been found in human tissue. These subclasses include the collagenases (MMP-1, -8, -13, -18) which degrade collagen, the gelatinases (MMP-2, -9) which degrade denatured collagen and type IV collagen, matrilysin (MMP-7) and the stromelysins (MMP-3, -10, -11), which degrade proteoglycans, metalloelastase (MMP-12) degrading elastin, and enamelysin (MMP-20) which degrades enamel. The structural classification is based on

specific protein domains that are involved in enzyme function. These domains include the haemopexin domain, which contains the inhibitor-binding site, and is present in all MMPs except MMP-7 and MMP-26. The fibronectin domain is important for the interaction with gelatin, and a furin-activated domain is involved in the process of intracellular activation. The membrane-type metalloproteases (MT-MMP) (MMP-14, -15, -16, -17, -24, -2) are unique in that they contain a GPI-anchored transmembrane domain, which locates the enzyme to the cell membrane (Nagase and Woessner, 1999), in contrast to all other MMPs which are secreted by the cell.

Most MMPs are secreted as inactive zymogens, which may be proteolytically activated by different proteinases such as other MMPs, plasmin, trypsin, chymotrypsin and also by the cathepsins B, L and G. The interaction between the MMPs and the tissue inhibitors of metalloproteases (TIMPs) represents another regulatory element in the cascade of protease action (Brew et al, 2000). TIMPs are specific endogenous inhibitors of MMPs and bind to the MMP molecule in a 1:1 stochiometric ratio (Yong et al, 2001). As of now, four different members have been identified, TIMP-1 to -4.

Another group of proteolytic metalloprotease is the ADAM family, which consists of at least 33 different enzymes. The functional structure of most members includes a pre-domain, a disintegrin domain, a metalloprotease domain and a transmembrane domain (Primakoff and Myles, 2000).

Recently, a new group of metalloproteases called the ADAMTSs was discovered. Like the ADAMs, these enzymes contain a disintegrin and metalloprotease domain. However ADAMTSs are characterized by the presence of additional thrombospondin type I (TSP-I) motifs in their C-terminal part, (Tang, 2001); 19 different members of ADAMTSs gene have been found. ADAMTSs are involved in BEHAB/brevican clevage by glioma cells (Matthews et al, 2000) in a way that separates the terminal globular domains and is likely to impair the ability of truncated B/b to anchor cells to the matrix. Not surprisingly, the activity of certain ADAMTS enzymes (Nakada et al, 2005) and therefore the levels of B/b cleavage fragments (Viapiano et al, 2005) are upregulated in gliomas compared with those in the normal brain. The molecular mechanisms by which B/b promotes glioma growth and dispersion are mostly unknown, although it is known that its overexpression does not affect glioma-cell proliferation or apoptotic rate in vitro (Nutt et al, 2001). Interestingly, the pro-invasive role of this CSPG in glioma seems to be a function of its cleavage products that is not shared with the full-length protein. Indeed, absence of ADAMTS-mediated cleavage renders B/b

unable to promote glioma invasion, but an N-terminal fragment of B/b is enough to promote invasiveness in vitro and in vivo. This specific pro-invasive role of a truncated isoform of B/b, absent in the full-length protein, illustrates how different molecular variants of a CSPG might mediate different, non-overlapping functions in the normal and diseased CNS.

In gliomas a number of MMPs have been implicated, but most studies have focused on matrix metalloproteinases MMP2 and MMP9, the serine protease urokinase-type plasminogen activator and its receptor, and the cysteine protease cathepsin B (Rao, 2003).

Immunohistochemical data have shown that MMP-2 and MMP-9 are highly expressed in gliomas although associated only with anaplastic astrocytomas and GBMs (Nakada et al, 1999). MMP-9 immunoreactivity is most intensely associated with endothelial vessels and not with tumor cells, likely reflecting the role in angiogenesis of GBM (Figure 2).

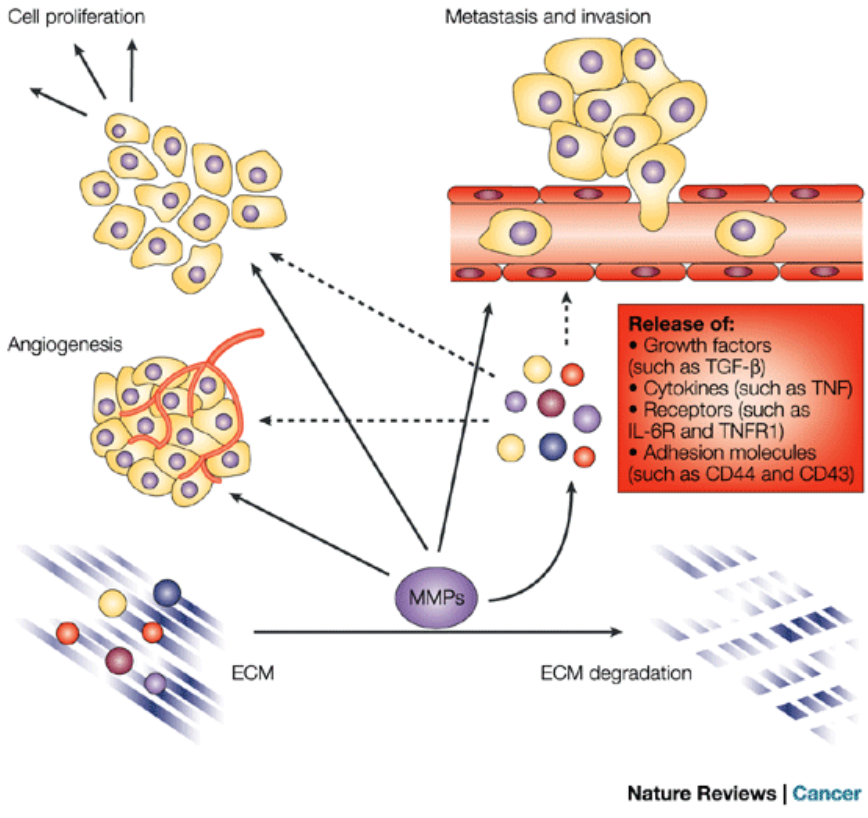

Figure 2: Matrix metalloproteinases (MMPs) promote growth of cancer cells through the interaction of extracellular matrix (ECM) molecules and integrins. MMPs promote angiogenesis by increasing the bioavailability of pro-angiogenic growth factors. MMPs also regulate invasion and migration by degrading structural ECM components. (Jasti S. Rao, 2003).

The membrane associated MT-MMPs has been found to be important in glioma progression and invasion in many studies. The expression of both MT1-MMP (MMP-14) and MT2-MMP (MMP-15), but not of that MT3-MMP (MMP-16), correlates with the grade of malignancy of a glioma (Nakada et al, 2001; Nakada et al, 1999). Cell invasion by MT1-MMP can be affected either indirectly through proteolysis of other factors or by a direct effect (Hotary et al, 2000). Although MT4-MMP (MMP-18) is present in the normal brain, its expression in gliomas is less certain (Vecchi et al, 1998). Higher expressions of MT5-MMP (MMP-24) (Liotta et al, 1991) and of MT6-MMP (MMP-25) (Velasco et al, 2000) were found in human brain tumours compared to normal brain; nevertheless, a more comprehensive analysis is required.

Complementing the increase in MMPs, the tissue inhibitors of MMPs (TIMP1 and 2) are expressed at low levels in malignant gliomas (Yamamoto et al, 1994). But others show an upregulation of TIMP-1 expression in malignant gliomas (Lampert et al, 1998; Nakano et al, 1995). Overexpression of TIMP-3 has been shown to suppress infiltration by glioma cells (Baker et al, 1999).

Other non-MMP proteases, including urokinase-type plasminogen activator (uPA) (Landau et al, 1994; Yamamoto et al, 1994) and cystein proteases (e.g., cathepsin B) (McCormick, 1993) are elevated in high-grade gliomas.

Expression of these proteases increases with glioma grade, and interference with the function of these proteases in vitro results in decreased invasive and/or migratory properties.

Most malignant gliomas express a variety of integrin receptors that mediate interactions with molecules in the extracellular space. The integrin heterodimers that have been most clearly implicated in glioma migration and invasion are α2β1, α5β1, α6β1, and αvβ3, which interact with tenascin, fibronectin, laminin, and vitronectin, respectively (Uhm et al, 1999). Activation of these integrins through interaction with extracellular ligands results in alterations of the cellular cytoskeleton, promoting locomotion. Attention has also focused on focal adhesion kinase (FAK) as a significant intermediate signaling molecule in glioma migration. FAK is a cytoplasmic tyrosine kinase expressed in highgrade gliomas and activated by EGFR and integrins such as αvβ3 and αvβ5. FAK subsequently signals through different pathways that affect proliferation, survival, and migration (Natarajan et al, 2003).

Gene expression profiling data further supports the notion that EGFR signaling contributes to glioma cell invasion. Overexpression of the vIII EGFR mutant (a constitutively active EGFR variant missing most of the extracellular domain)

results in upregulated expression of multiple genes associated with invasion, including metalloproteinases (MMP1 and MMP13) and collagens (Lal et al, 2002). Furthermore, in glioma invasion model systems, inhibition of EGFR appears to inhibit invasion (Penar et al, 1997).

Necrosis, Hypoxia and Angiogenesis

The known molecular changes underlying malignant progression are similar among the different types of glioma. The progression from WHO grade II to anaplastic, WHO grade III diffuse glioma is associated with a marked increase in clinical aggressiveness. At a histological level, grade III tumors have increased cellularity and mitotic activity that is usually identifiable by light microscopy, as well as proliferative indices substantially above those of grade II tumors.

Necrosis is a central feature of the highest grade malignant gliomas (Randy L. Jensen, 2009). In response to hypoxia, small clones of tumor cells acquire molecular characteristics that encourage more active migration, thus clearing a central region more susceptible to necrosis. This hypothesis is based on work showing that glioblastoma tumor cells surrounding necrotic centers (often forming the so-called pseudopalisades) are less proliferative and more apoptotic than adjacent cells (Brat et al, 2004). These perinecrotic cells also express hypoxia-inducible genes, such as hypoxia-inducible factor 1 alpha (HIF1α) (Brat et al, 2004), and in vitro studies have demonstrated that hypoxia increases cellular migration and gelatinase activity. Morphometric analysis of the central necrotic zones of pseudopalisades in glioblastomas demonstrate abnormal and sometimes thrombosed vessels in more than half, suggesting that an initial vaso-occlusive event initiated the hypoxia/necrosis cycle. It is also possible that the endothelial cells of small vessels are driven toward apoptosis and vascular regression as a result of angiopoietin-2 expression (Holash et al, 1999) (Figure 3).

Interactions between hypoxia, necrosis, upregulation of growth factors, and selection of malignant clones begins to provide a simple explanation for the marked histological heterogeneity noted in glioblastomas and a possible explanation for the marked resistance to conventional cytotoxic therapies seen in clinical patients with glioblastoma.

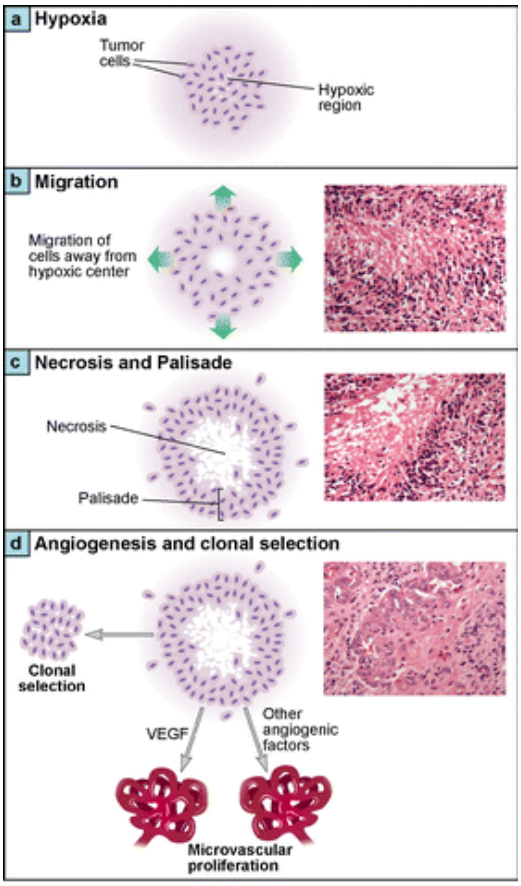

Figure 3: Necrosis/hypoxia and angiogenesis. (a) Localized hypoxia appears to upregulate migration-associated genes, (b) leading to migration of tumor cells away from a central hypoxic center. (c) Necrosis then ensues in the central region, sometimes in association with vascular thrombosis, and a palisade of densely packed tumor cells develops. These palisading cells express abundant angiogenic factors such as vascular endothelial growth factor (VEGF), which leads to (d) adjacent angiogenesis that includes so-called glomeruloid microvessels (Louis, 2003).

The most characteristic form of vascular proliferation, however, is microvascular proliferation in the form of so-called glomeruloid vessels (named after their resemblance to renal glomeruli). These vascular formations are unique to high-grade gliomas; for example, nearly all diffuse astrocytic tumors with microvascular proliferation are classified as glioblastomas, and nearly all oligodendroglial tumors with microvascular proliferation are classified as anaplastic oligodendrogliomas.

The biological underpinnings of vascular proliferation in malignant gliomas are complex, with many angiogenic growth factors and their receptors occuring in glioblastomas (Cavenee et al, 2000). For example, VEGF and PDGF are expressed by tumor cells whereas their tyrosine kinase receptors, VEGF receptors 1 and 2 and PDGFβ-receptor, are expressed on endothelial cells. VEGF and its

receptors, in particular, appear to play a major role in glioblastoma angiogenesis (Plate et al, 1992; Brat and Van Meir, 2001). VEGF and its receptors, which cause vascular permeability, may also be responsible for breakdown of the blood brain barrier and tumoral edema in glioblastoma. VEGF is secreted by tumor cells and bound by the VEGF receptors on endothelial cells. VEGF is a downstream target of a number of the activated signaling pathways in glioblastomas. Angiogenesis in solid tumors, including brain tumors, is believed to be triggered by hypoxia. Exposure of brain tumor cells to hypoxia induces expression of Hif-1 that activates transcription of VEGF and other proangiogenic factors in gliomas (Shweiki et al, 1992). Researchers have found high levels of expression of VEGF mRNA in the hypoxic regions of high-grade but not low-grade gliomas.

In addition, the VEGF receptors (VEGFR1 and VEGFR2) are highly expressed in gliomas (Plate et al, 1992); bFGF is a potent mitogen of endothelial cells and is required for glioma angiogenesis in vivo (Auguste et al, 2001).

The intracellular machinery that governs the signaling from the receptors on the cell surface to the nucleus to control induction of angiogenesis remains poorly understood. Signaling of VEGFR and that of other receptor tyrosine kinases, such as the platelet-derived growth factor receptors (PDGFRs) and epidermal growth factor receptors, have regulatory mechanisms that are similar in many aspects (Olsson et al, 2006). VEGFR signaling may induce activation of Ras/Raf/mitogen-activated protein kinase (Takahashi et al, 1999) or phospholipase C-γ/protein kinase C signaling,which regulates endothelial cell proliferation, migration, and permeability (Lal et al, 2001). Also, one of the important signaling pathways activated by VEGFR is the phosphatidylinositol-3 kinase/phosphatase and tensin homologue (PTEN)/Akt/mammalian target of rapamycin (mTOR) pathway. This PI3K/ PTEN/mTOR pathway regulates endothelial cell survival, translation, and permeability (Takahashi et al, 2003). This pathway is also activated by other proangiogenic stimuli, including PDGF, neurotrophins, IGF, epidermal growth factor, and integrins, and plays a critical role in brain tumor angiogenesis (Castellino and Durden, 2007). The role of this signaling pathway in the proliferation and survival of brain tumor cells strongly suggests the potential use of inhibitors of it to target both brain tumor cells and blood vessel endothelial cells.

The blood-brain barrier in brain tumors

The vasculature in a healthy central nervous system (CNS) tissue is highly specialized and distinguished from the vasculature in other tissues by a unique structure of blood capillaries, the blood-brain barrier (BBB) (Deeken et al, 2007). Unlike other tissues, in which relatively free diffusion of materials in the blood is allowed through their peripheral capillary walls, the transportation of materials in the blood circulation to the peripheral tissues in CNS is tightly regulated by this barrier. The BBB is an anatomical and physiological barrier that strictly restricts the permeability of blood vessels, suppressing the diffusion of ions, peptides, amino acids, and other substances from the bloodstream to the neural system while supplying the brain with the required nutrients for proper CNS function. This barrier is composed of the walls of vessel endothelia, which are sealed by tight junctions between endothelial cells (Figure 4). Also, the BBB is wrapped with specialized cells (pericytes) and the flattened "end feet" of astrocytes. Pericytes are relatively undifferentiated mesenchyme-like cells that support capillary blood vessels. Astrocytes induce the tight junctions of BBB through decreasing VEGF expression and stimulating angiopoietin release (Lee et al, 2003).

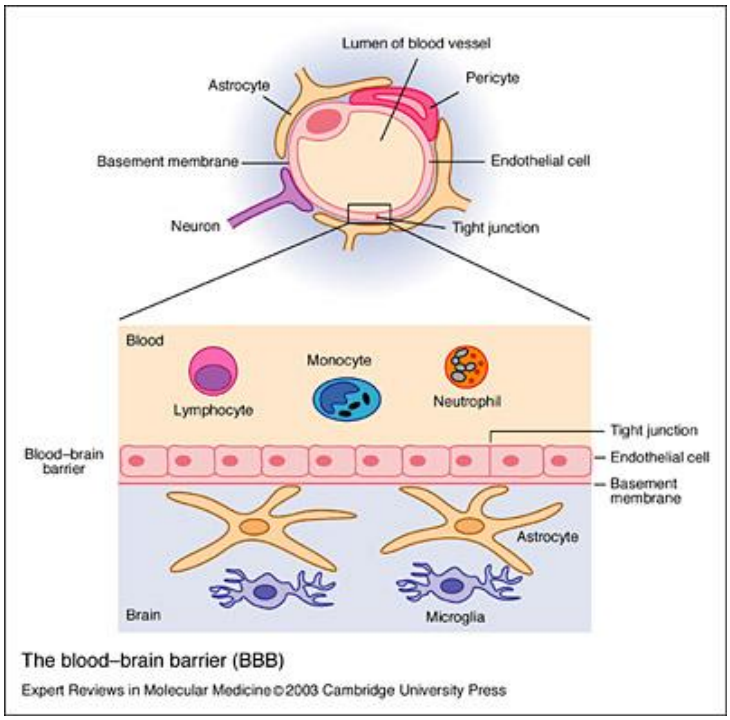

Figure 4: The blood barrier is created by the tight apposition of endothelial cells lining blood vessels in the brain preventing easy passage of large macromolecules and pathogens between the circulation and the brain.

The BBB in brain tumors is structurally and functionally abnormal (Fukumura et al, 2001; Yuan et al, 1994). Nevertheless, some features of the normal BBB are retained in brain tumor vasculature (Monsky et al, 2002). Researchers found that, in a murine brain tumor metastasis model, the integrity of the BBB was conserved in small tumors (<0.25 mm in diameter) but not in larger tumors (Fidler et al, 2002). In addition to loss of BBB integrity, blood vessels in brain tumors exhibit abnormal features similar to those in the vessels in other types of tumors. For example, tumor blood vessels are tortuous, disorganized, and highly permeable because of abnormalities in their endothelial walls (Bullitt et al, 2005). Therefore, disruption of the BBB and further increases in the permeability of tumor blood vessels in brain tumors because of their loosened endothelial structures result in increased accumulation of fluid peritumorally and in the surrounding brain and bring about vasogenic brain edema. Vasogenic edema is a major cause of morbidity in patients with brain tumors (Jain et al, 2007; Jain et al, 2007). Tumor angiogenesis must be treated properly not only to prevent brain tumor growth but also to suppress the pathological damage caused by changes in the permeability of brain tissue. The blood vessels associated with brain metastases are dilated and contain highly mitotic endothelial cells, which may require high concentrations of VEGF for their growth. Increased leakage from blood vessels in brain tumors causes suppressed and irregular blood flow and leads to heterogeneous and inefficient delivery of oxygen, nutrients, and drugs to the brain tumor via the bloodstream (Grizzi et al, 2005).

One of the main causes of increased permeability and loss of BBB integrity in brain tumor blood vessels is increased expression of VEGF by the brain tumor cells. Investigators initially purified VEGF for its ability to induce vascular leakage and permeability as well as for its role as a mitogenic factor for endothelial cells. Therefore, it was originally known as vascular permeability factor as well as VEGF (Murohara et al, 1998). The effects of VEGF on vascular permeability in the peripheral circulation appear to occur via modulation of calcium influx, nitric oxide; activation of guanylyl cyclase, protein kinase G, vesiculo-vacuolar organelles, or increased synthesis of platelet-activating factor in endothelial cells (Mayhan, 1999). In principle, tumor blood vessels, especially brain tumor blood vessels, are abnormal, which means they are highly permeable and even leaky at many points, resulting in irregular and inefficient blood flow through them. This irregularity and inefficiency are strongly linked with the action of VEGF, the major proangiogenic factor (Jain et al, 2007).

Apoptosis

Apoptosis is a morphologically defined mode of cell death characterized by nuclear condensation and fragmentation, membrane blebbing and formation of apoptotic bodies and subsequent phagocytosis of these vesicles by surrounding cells (Kerr et al, 1972). All of these features distinguish apoptotic cell deaths from necrotic cell deaths which are commonly induced by external trauma in its broadest sense (mechanical trauma, heat, bacterial inflammation, and others) and affect continuous tissues rather than single cells within tissues.

Apoptotic process can be triggered via extrinsic and intrinsic apoptotic pathways (Figure 5).

The extrinsic apoptotic pathway is activated by the immune system following detection of abnormal surface markers on the cell. There is subsequent release of specific ligands that bind and activate cell surface death receptors (DR) which include the Fas and TNF receptor families. This results in intracellular signal transduction through the formation of a death-inducing signalling complex, causing activating cleavage of caspases 8 and 10. These in turn cleave caspases 3 and 7, which go on to begin the irreversible apoptotic cascade (Degterev et al, 2003).

The intrinsic apoptotic pathway is triggered by direct damage to the cell from various insults, including hypoxia, oxidative stress, radiotherapy and chemotherapy, all of which can cause influx of Ca2+ into the cell, generation of ROS and direct DNA damage. As a result, p53 is activated and subsequently enhances DNA transcription of anti-proliferative and pro-apoptotic genes (Klein et al, 2005). The up-regulated pro-apoptotic gene products include p53 upregulated modulator of apoptosis (PUMA), phorbol-12- myristate-13-acetate-induced protein 1 (NOXA) and BCL-2 homology domain 3 proteins (BH3), which interact with the B cell lymphoma-2 (BCL-2) homology family of proteins. The members of this family have a close structural and functional relationship with the mitochondrial membranes and exert either membrane-stabilising or permeability inducing effects, classifying them into anti-apoptotic and pro-apoptotic, respectively. PUMA, NOXA and BH3 stimulate the pro-apoptotic BCL-2 family members and, together with these, inhibit the anti-apoptotic family members (O'Neill et al, 2004). It is believed that the resulting shift in balance towards apoptosis causes opening of a mitochondrial permeability transition pore (MPTP) through the interaction of several proteins. This pore allows the rapid release of cytochrome C (Cyt C), second mitochondria-derived activator of

caspases (SMAC) and Omi from the mitochondrion into the cytosol. Cyt C then binds with apoptotic peptidase activating factor-1, a p53 enhanced gene product, and caspase 9 to form an apoptosome which cleaves caspases 3 and 7, inducing the apoptotic cascade, whilst SMAC and Omi inhibit the function of the inhibitors of apoptosis (IAP) family of proteins which prevent apoptosome action (Kroemer et al, 2007).

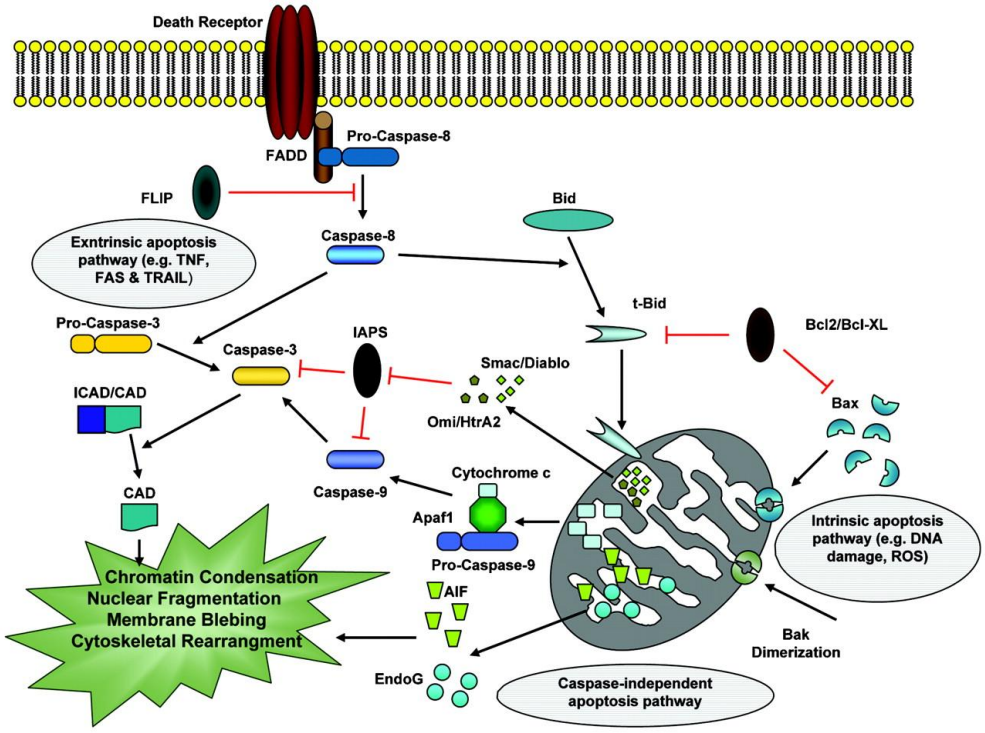

Figure 5: The molecular mechanisms of apoptosis. Apoptosis pathways can be initiated via different stimuli—that is, at the plasma membrane by death receptor ligation (extrinsic pathway) or at the mitochondria (intrinsic pathway) (Ghavami et al, 2009).

There are no specific features of apoptosis in glioma cells compared with apoptosis in other cell types. Moreover, the major molecular players of the apoptotic process are largely the same in glioma cells, other cancer cells and non-transformed glial and non-glial cells.

p53 is a cell cycle control protein which senses genotoxic stress leading to DNA damage and arrests cells in GO/G1 or G2/M, or induces their apoptosis. The molecular consequences of p53 activation include changes in the transcription of various p53 response genes, including p21 and BAX, as well as transcription-independent effects which are, however, less well-defined. p53 may be the most common target gene for mutational inactivation in human cancers. p53 mutations are rather common (65%) in secondary glioblastomas thought to be derived

through the malignant progression from grade II or III astrocytomas. In these patients, the same p53 mutations are already found in the less malignant precursor lesion in approximately 90% (Watanabe et al, 1997). In contrast, only 10% of primary glioblastomas exhibit p53 mutations. However, within the process of neoplastic transformation, the loss of p53 probably allows the cell to accumulate random genetic and chromosomal aberrations without triggering the endogenous p53- controlled cell death pathway. Further, naturally occurring mutations of p53 often result in unpredictable biological effects which include gain-of-function properties of mutant p53.

BCL-2 family proteins comprise a large family of proteins which share a BCL-2 homology (BH) region, interact by heterodimerization and either inhibit or promote apoptosis. Human glioma cell lines express a variety of antiapoptotic and proapoptotic BCL-2 family proteins (Weller et al, 1998). In contrast to the expectation that the expression of antiapoptotic members of the BCL-2 family increases with malignancy, stronger staining in astrocytoma and anaplastic astrocytoma compared with glioblastoma has been observed in most (Krajewski et al, 1997), but not all studies. An up-regulation of BCL-2 and BCLXL, but a down-regulation of BAX has been described in recurrent glioblastoma. Overexpression of BCL-2 or BCL-XL induces complex changes of the glioma cell phenotype in that it not only protects glioma cells from various proapoptotic stimuli (Glaser et al, 1999) but also enhances their motility (Wick et al, 1998).

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is normally expressed on both normal and tumour cells as a non-covalent homotrimeric type-II transmembrane protein (memTRAIL). In addition, a soluble form of TRAIL (sTRAIL) can be generated due to alternative mRNA splicing (Bouralexis et al, 2005) or proteolytic cleavage of the extracellular domain of memTRAIL (Wiley et al, 1995) and thereby still retaining tumourselective pro-apoptotic activity. TRAIL has an intricate receptor system comprising four distinct membrane receptors, designated TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4. Of these receptors, only TRAIL-R1 and TRAIL-2 transmit the apoptotic signal. These two receptors belong to a subgroup of the TNF receptor family, the so-called death receptors, and contain the hallmark intracellular death domain (DD). This DD is critical for apoptotic signalling by death receptors. TRAIL activates the extrinsic pathway of apoptosis by binding to TRAIL-R1 and/or TRAIL-R2, whereupon the adaptor protein Fas-associated death domain and initiator caspase-8 are recruited to the DD of these receptors. Assembly of this so-called death-inducing signalling complex leads to the sequential activation of initiator and

effector caspases, and ultimately results in apoptotic cell death.

In certain cells, the execution of apoptosis by TRAIL further relies on an amplification loop via the intrinsic mitochondrial pathway of apoptosis by Bcl-2 homology (BH3) only protein Bid that is cleaved into a truncated form (tBid) by active caspase-8. Truncated Bid subsequently activates the mitochondrial pathway. TRAIL-R3 is a glycosylphosphatidylinositol-linked receptor that lacks an intracellular domain, whereas TRAIL-R4 only has a truncated and non-functional DD. The latter two receptors are thought to function as decoy receptors that modulate TRAIL sensitivity; however, the mechanism underlying this decoy function is not yet elucidated.

Previously, it was reported that TRAIL mRNA transcription is detectable in normal brain tissue; however, it was not clearly specified if this was neuronal or glial tissue (Daniels et al, 2005).

Like TRAIL also TRAIL death-inducing receptors (TRAIL-R1/R2) are expressed on many normal tissues (Spierings et al, 2004). Vascular brain endothelium appears to be negative for TRAIL-R1 and weakly positive for TRAIL-R2. TRAIL-R2 and TRAIL-R4 have been detected on oligodendrocytes and neurones. TRAIL-R1 have been detected on astrocytes (Dorr et al, 2002).

TRAIL-R1 and TRAIL-R2 are ubiquitously expressed on a variety of tumour types (McCarthy et al, 2006). Importantly, TRAIL-R1 andTRAIL-R2 are also expressed in the tumour tissue from astrocytoma grade II and glioblastoma patients (Frank et al, 1999). In a study on 62 primary GBM tumour specimens, TRAIL-R1 and TRAIL-R2 were expressed in 75% and 95% of the tumours, respectively. Of note, a statistically significant positive association was identified between agonistic TRAIL receptor expression and survival (Kuijlen et al, 2006). Interestingly and perhaps counter-intuitively, highly malignant tumours actually express a higher amount of TRAIL receptors in comparison with less malignant tumours or normal tissue. In general TRAIL-R2 is more frequently expressed on tumour cells than TRAIL-R1. Several studies in GBM cell lines were unable to correlate TRAIL sensitivity to the expression levels of the agonistic TRAIL receptors TRAIL-R1 or TRAIL-R2 nor to the expression levels of the decoy receptors TRAIL-R3 and TRAIL-R4 (Knight et al, 2001; Hetschko et al, 2008).

MICROVESICLES

The release of small vesicles by many cell types as tools for intercellular communication was proposed a few decades ago (Bastida et al, 1984) and has recently acquired further validation in several systems. It was previously thought that communication between cells was mediated only by molecules, such as neurotransmitters and hormones, released (or surface-exposed) by one cell and deciphered by the other, upon receptor binding. Discrete vesicles frequently observed through electron microscopy in the intercellular space were assumed to result from damage, such as necrosis or apoptosis that form apoptotic bodies (ABs), of the surrounding cells. An understanding of the nature of these structures changed when the release of small vesicles was recognized to result from specific processes (Beaudoin and Grondin, 1991).

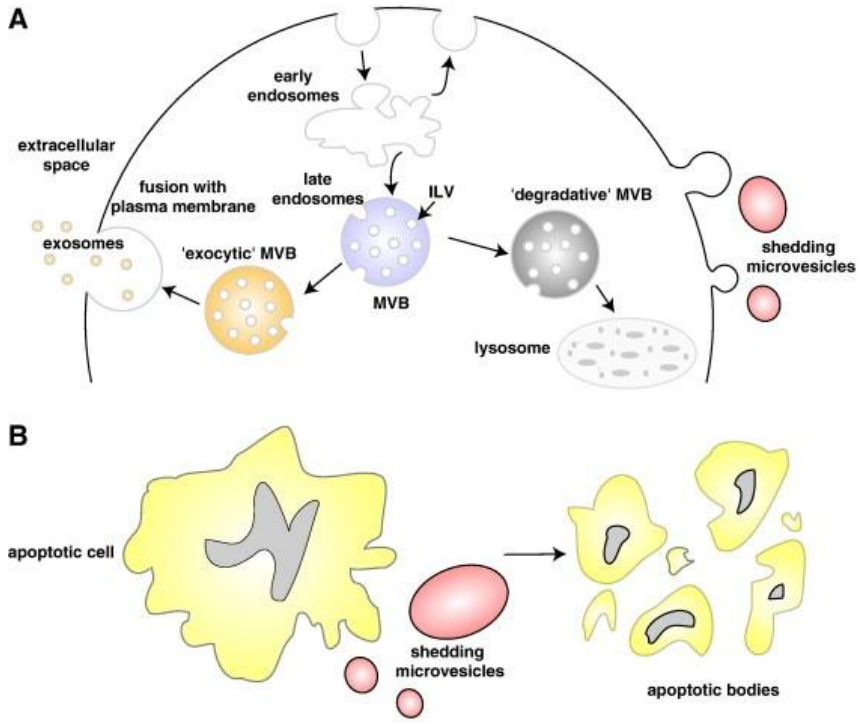

Figure 6: Schematic representation of the release of extracellular membrane microvesicles into the extracellular space. (A) Release of exosomes and SVs is shown. (B) Apoptotic or dying cells with cell shrinkage, a hallmark of apoptosis, cause generation of ABs. These vesicles are remnants of the degrading apoptotic cell with nuclear and cytoplasmic content (Mathivanan et al, 2010).

Two such vesicle-discharge processes were identified, each leading to the release of distinct types of signaling vesicles: i) exocytosis of multivesicular bodies (MVBs), intracellular organelles of the endosomal system that contain exosomes,

vesicles with a diameter of 30 nm - 100 nm, released from the cell; ii) direct budding from the plasma membrane of small vesicles, called shed vesicles (SVs), 100 nm – 1 μm in diameter, released into the extracellular space. Mixed vesicle populations, containing both shedding vesicles and exosomes, will be referred to as microvesicles (MVs) (Figure 6).

Shed vesicles

SVs and exosomes are morphologically distinct. A recent study revealed that protease-containing SVs from tumor-cell lines appear to be rather heterogeneous both in size, ranging from 200 nm to more than 1 μm in diameter, and shape, as opposed to exosomes, which range from 50 nm to 100 nm in diameter and form a more uniform population of vesicles (Muralidharan-Chari et al, 2009). The same study also showed that SVs sediment at lower speed relative to exosomes, which pellet at 100,000 g.

Much remains to be understood about the mechanisms by which SVs are formed and shed at the cell surface. It appears that the release of the SVs population initiated by outward budding from the surface of the plasma membrane is followed by a fission event that in many ways resembles the abscission step in cytokinesis. The release of SVs also appears to share similarities with the events associated with viral budding (Chazal and Gerlier, 2003; Morita and Sundquist, 2004).

Moreover, there are structural similarities between membrane-derived shed vesicles and apoptotic blebs, both of which are formed by outward protrusion of the plasma membrane. Unlike apoptotic bodies, however, shed vesicles do not contain cytosolic organelles and/or nuclear fragments. Consistent with this understanding is the knowledge that multiple cell lines of both normal and transformed origin remain viable in culture even when stimulated to release SVs (Dolo et al, 1998; Ginestra et al, 1998; Taraboletti et al, 2002). A recurring theme, however, is that the initiating events involve both lateral and vertical redistribution of plasma membrane constituents resulting in alteration of the local membrane curvature.

SVs shedding has also been shown to take place at specific locations on the cell membrane that are enriched in assorted lipids and proteins, with the exact composition reflecting the cellular origin (Figure 7). Common among these lipid requirements is cholesterol, which is a key component of membrane lipid rafts.

Lipid rafts were previously hypothesized to have a role in the initial 'pinching' events, because the SVs released from activated neutrophils contained high levels of cholesterol, and pharmacological depletion of cellular cholesterol inhibits the shedding (Del Conde et al, 2005; Pilzer et al, 2005). Similarly phosphatidylserine has been found to be exposed on the extracellular leaflet of shed vesicles (Muralidharan-Chari et al, 2009). This topological reversal may serve several purposes. First, the packing defects that result from the addition of the aminophospholipid to the extracellular leaflet can cause shape changes in the plasma membrane. Second, it could promote detachment of the membrane from the underlying cytoskeleton. Functionally, exposure of phosphatidylserine on the outer leaflet also allows the shed vesicle to become a target for the immune system.

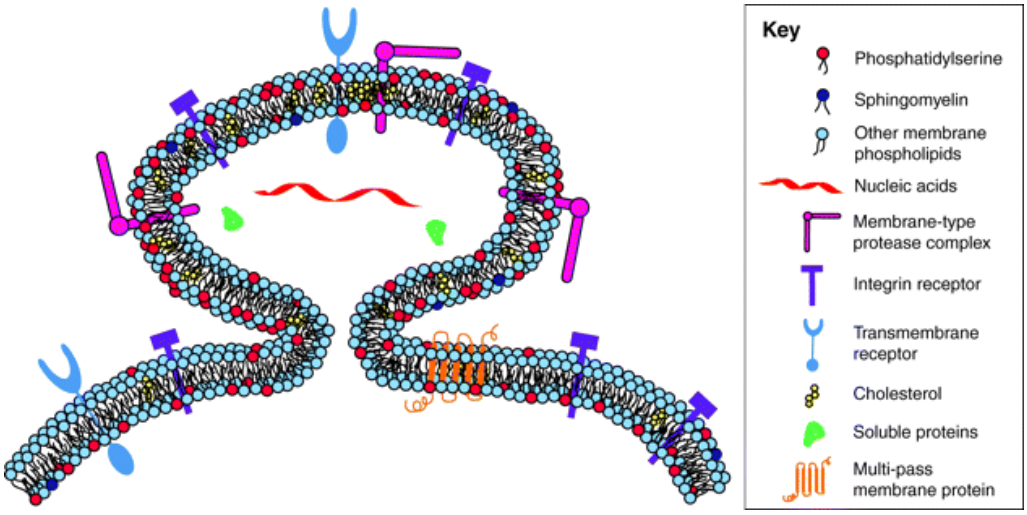

Figure 7: Microvesicles are formed by the outward budding of the plasma membrane. Not all plasma-membrane proteins are incorporated into shed vesicles, although the topology of membrane proteins remains intact. Microvesicles appear to be enriched in some lipids such as cholesterol, whereas phosphatidylserine is relocated to the outer membrane leaflet specifically at sites of microvesicle shedding (Muralidharan-Chari et al, 2010).

Concomitant with the assorted modes of microvesicle initiation, formation and release, there are also multiple signaling pathways that are thought to regulate their biogenesis. There are many reports that highlight a potential role for growth factors in cell activation and subsequent SVs release, because removal of serum from the growth medium abolishes the release (Vittorelli, 2003). There is also substantial evidence of increased shedding activity in microglia and dendritic cells when stimulated with Ca2+ (Cocucci et al, 2009). However, Ca2+ is not the only

second messenger involved. In various cell types, in fact, phorbol ester activation of protein kinase C (PKC) is also effective (Sidhu et al, 2004). Energy input is also required for vesicle release. In PC12 cells, shedding vesicles are released upon application of phorbol esters but not of Ca2+ ionophores (Cocucci et al, 2007). The purinergic receptors of ATP, a ligand released by many cell types, have an important role. In dendritic cells, macrophages and microglia, activation of the purinergic receptor-channel, P2X7, was found to induce intense release (Bianco et al, 2005). In other cell types (such as PC12 and platelets), activation of the P2Y receptors coupled with the Gq protein was found to be effective (Kahner et al, 2008).

Exosomes

The exosomes were first identified as the vesicular structures segregated within a ubiquitous membrane-bound organelle, the MVB. MVBs were first discovered in the early days of electron microscopy. For a long time, however, they were interpreted only as a late step in the maturation of endosomes to lysosomes. Approximately 20 years ago, however, MVBs were shown to also participate in an alternative process, the exocytic fusion of their external membrane with the plasma membrane of the cell, with ensuing discharge of their segregated vesicles to the extracellular space. The generation of exosomes, with budding, fission and segregation occurring within the MVB lumen, does not occur by default but is governed by specific processes. Well characterized multiprotein complexes, the Endosomal Sorting Complexes Required for Transport (ESCRTs), have been shown to play a key role in the accumulation of vesicles addressed to lysosomal degradation (Hurley, 2008).

The ESCRT-0, -I and -II complexes recognize and sequester ubiquitinated proteins in the endosomal membrane, whereas the ESCRT-III complex seems to be responsible for membrane budding. While the ESCRT proteins, which were first identified in yeast genetic screens for vacuolar protein sorting (vps) mutants, are required for the targeting of membrane for lysosomal degradation, the function of the ESCRT machinery in the formation of intraluminal vesicles (ILVs) that are further secreted as exosomes is less clear. There is still no clear evidence whether the sorting of selected exosomal cargo requires ESCRT function. Proteomic analyses of purified exosomes from various cell types show an enrichment of different ESCRT components and ubiquitinated proteins (Buschow et al, 2005).

Moreover, the expression of the Nedd4 family interacting-protein 1 is associated with elevated levels of protein ubiquitination in exosomes (Putz et al, 2008), and Alix, a protein that associates with the ESCRT machinery, is required for the sorting of the transferrin receptor into exosomes (Geminard et al, 2004). These observations lead to the hypothesis that ESCRT function could be required for sorting of exosomal cargo.

However, recent studies have provided evidence that some exosomal proteins are released in an ESCRT-independent manner (Trajkovic et al, 2008). One of these pathways requires ceramide (Bianco et al, 2009), and the endosome sorting into the exocytic MVBs, in an oligodendrocyte cell line (Trajkovic et al, 2008). Ceramide has many structural and physical properties that may facilitate vesicle biogenesis. Ceramides are known to induce lateral phase separation and domain formation in model membranes (Goni and Alonso, 2006). In addition, the cone-shaped structure of ceramide may induce spontaneous negative curvature in the membrane bilayer promoting membrane invagination. Proteins, such as tetraspanins, may be partitioned into these domains. It is also possible that tetraspanins are part of the sorting system as they are known to form oligomers by interacting with other tetraspanins and also with a variety of transmembrane and cytosolic proteins (Zoller, 2009).

Exosomes are now understood in much more detail than shed vesicles (Schorey and Bhatnagar, 2008).

There is accumulating evidence that MVBs are more heterogeneous. It is clear that there must be different kinds of MVBs – one class that eventually ends up in lysosomes and another class that fuses with the plasma membrane to release exosomes – but methods to separate these subpopulations are not available. Whether these MVBs contain intermixed populations of ILVs or specific set of ILVs destined either for the release as exosomes or for delivery to lysosoems is not known.

Multiple pathways seem, thus, to operate on endosomal membranes and give rise to different populations of MVBs and ILVs. An important goal is to understand if the different populations of MVBs emerge together to finally segregate from each other or if they originate independently. It would be equally important to be able to define the different types of MVBs not only on the basis of their morphology but also of their composition (Figure 8). These distinct MVBs require different mechanisms to regulate fusion with the target membrane. While the largest population of MVBs continuously fuse with lysosomes in most cells (Luzio et al, 2007), the fusion events are prevented in some instances, as MVBs can also house

proteins for temporary storage. Little is known about the requirements and regulation of MVB fusion with the plasma membrane. As for lysosomal secretion, calcium ionophores stimulate exosome release in some cell types, suggesting that intracellular calcium levels play a role in plasma membrane fusions event (Savina et al, 2003). However, the fusion machinery regulated by Ca2+ remains undefined. The small GTPase Rab11 and the citron kinase, a RhoA effector, may participate in the exocytic event (Savina et al, 2005). A potential candidate that has been proposed to mediate fusion of MVBs with the plasma membrane is the V0-subunit of the V-ATPase. Independently of the V0-V1 ATPase complex, the V0-subunit plays a role in membrane fusion (Marshansky and Futai, 2008) and a V0- ATPase mutant in Caenorhabditis elegans impairs fusion of MVBs with the apical membrane of the cuticles (Liegeois et al, 2006).

There are also conditions in which fusion of MVBs with the plasma membrane is prevented. For example, induction of autophagy inhibits exosome secretion and promotes the fusion of MVBs with autophagic vacuoles (Fader et al, 2008).

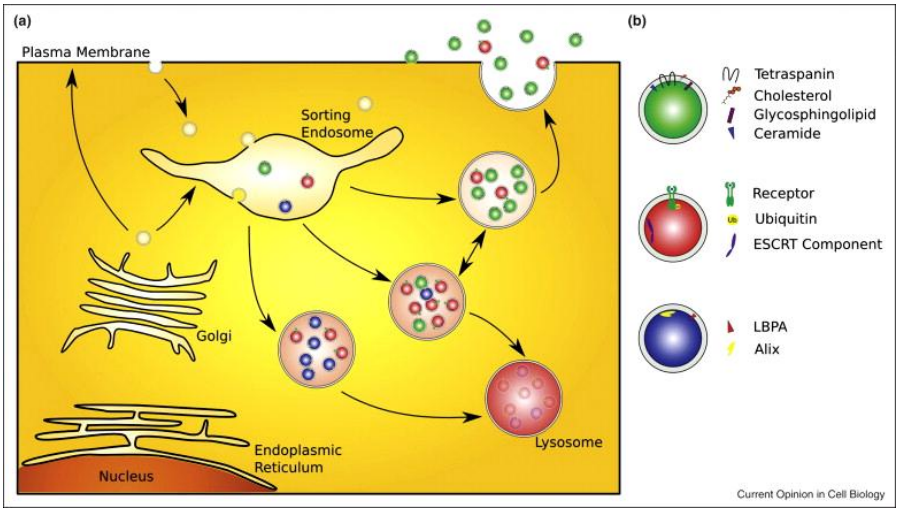

Figure 8: Model for sorting of cargo into different MVB subpopulations. (a) Different hypothetical MVB subclasses with distinct populations of ILVs (red, green and blue) are shown. The putative compositions of these ILVs are shown in the right panel. (b) At least three different subclasses of ILVs may coexist. The molecules shown represent a selection of protein and lipids that define different classes of ILVs (Simons and Raposo, 2009).

MVs in the extracellular space

Upon release, both types of microvesicles circulate in the extracellular space adjacent to the site of discharge where they can be broken down, often within a

few minutes. Some of them, however, can move some distance by diffusion and can appear in biological fluids, such as cerebrospinal fluid, blood and urine. In terms of signaling, although a few general properties of microvesicles seem to be analogous to those of soluble agents, many others are distinct and more complex. In some cases, including the interaction between microglia and astrocytes, microvesicles deliver transmembrane signals which activate surface receptors (Bianco et al, 2005); in other cases, such as coagulation or inflammation, they function not only as messengers, but also as platforms necessary for the coordinate development of multisignaling processes (Del Conde et al, 2005). Being membrane-bound structures, microvesicles can receive retrograde signals and fuse with or are taken up by their target cells. Moreover, these microvesicles function in signaling and in the horizontal transfer of their membrane and/or cargo molecules, which are enriched in specific proteins and mRNAs. By this transfer, microvesicles can be important for the metabolism of target cells (Deregibus et al, 2007) (Figure 9). Owing to these many properties, microvesicles are involved in the control of coordinate processes, which are important for the general homeostasis of cell populations, in physiological as well as in many pathological conditions.

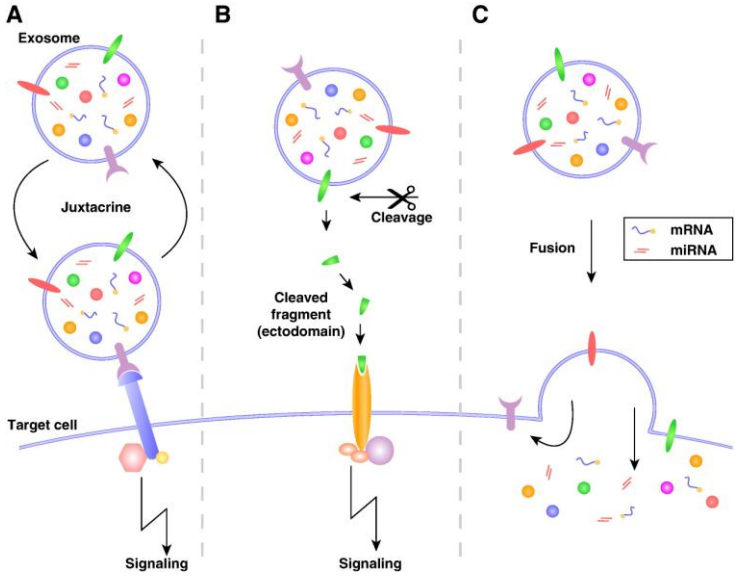

Figure 9: Possible mechanisms of intercellular communication by MVs. (A) Membrane proteins can interact with receptors in a target cell and activate intracellular signaling (juxtacrine). (B) Exosomal membrane proteins can be cleaved by proteases in the extracellular space. Cleaved fragments can then act as a soluble ligand which binds to the target cell surface receptor. (C) Exosomes can fuse with the target cell membrane and release their contents inside the recipient target cell in a non-selective manner (Mathivanan et al, 2010).

MVs in Tumor progression

The microenvironment of many tumors is highly enriched in vesicles shed not only by the proliferating cells but also by macrophages and neutrophils infiltrating the interstitium. Matrix digestion favors angiogenesis, which is of key importance for tumor growth and which is also stimulated by TF generated in the course of coagulation via the upregulation of the proangiogenic vascular endothelial growth factor. The increased vascularization is important to the migration of metastatic cells and it also improves malignant cell survival. Angiogenic effects are also induced by the horizontal transfer of mRNAs and miRNAs via vesicles shed by tumor cells and taken up by endothelial cells. Another mechanism by which shed vesicles can reinforce tumor cell growth is by triggering a Fas-dependent apoptosis of activated lymphocytes. Finally, accumulation of drugs, such as doxorubicin and other anti-cancer agents in the membrane of the shed vesicles decreases their cellular levels and can thus contribute to the process of drug resistance.

Microvesicles in cancer patients were first documented in 1978, when they were identified in cultures of spleen nodules and lymph nodes of a male patient with Hodgkin disease (Friend et al, 1978). About a decade later, it was demonstrated that plasma-membrane-derived vesicles shed spontaneously from highly metastatic B16 mouse melanoma (F10) cells and, when fused with weakly metastatic B16 mouse melanoma (F1) cells, enabled F1 cells to metastasize to the lung (Poste and Nicolson, 1980). Both of these studies set the stage for further investigations into the significance of microvesicle shedding in cancer progression. Since then, microvesicle-mediated cargo transfer to adjacent or remote cells has been shown to affect many stages of tumor progression (van Doormaal et al, 2009), including angiogenesis, escape from immune surveillance, ECM degradation and metastasis.

MVs and tumor angiogenesis

Angiogenesis is vital for tumor survival and growth, and occurs by proliferation of endothelial cells to form a mesh of tumor-infiltrating blood vessels that facilitate the supply of nutrients and oxygen for tumor growth as well as removal of waste products (Carmeliet, 2005). As discussed below, several reports indicate that tumor-derived microvesicles stimulate secretion of several pro-angiogenic

factors by stromal fibroblasts, and chemoattract and facilitate proliferation of endothelial cells to promote angiogenesis and enable tumor growth.

Matrix reorganization by endothelial cells, a cellular process that is facilitated by matrix-degrading proteases, particularly MMPs, is crucial for the process of vascularization under normal conditions and also in cancer. Dolo and colleagues showed that microvesicles shed by endothelial cells contain MMPs, such as MMP2, MMP9 and MT1-MMP, that facilitate autocrine stimulation of endothelial-cell invasion into Matrigel and result in cord formation (Taraboletti et al, 2002). In a follow-up study, the same group demonstrated that microvesicles isolated from ovarian-cell lines, such as CABA1 and A2780, stimulated the motility and invasiveness of endothelial cells in vitro and also reported the presence of VEGF in microvesicles together with MMPs (Taraboletti et al, 2006). Interestingly, Al-Nedawi and colleagues showed that the onset of VEGF expression and its receptor VEGFR in endothelial cells ensued following the transfer of EGFR via microvesicles shed by human cancer-cell lines that harbor the activated EGFR mutation (Al-Nedawi et al, 2009). Beside growth factors and proteases, microvesicle-mediated transfer of miRNAs has also been shown to stimulate tubule formation in endothelial cells, by modifying the translational profile of these cells and, thereby, promoting acquisition of the angiogenic phenotype (Skog et al, 2008).

Lipids from microvesicles can impact endothelial-cell migration and angiogenesis. In this regard, sphingomyelin, a major component identified in microvesicles shed from the fibrosarcoma cell line HT1080, together with VEGF, was shown to confer migratory and angiogenesis-inducing properties to endothelial cells (Kim et al, 2002). Whereas purified sphingomyelin elicited similar migratory and angiogenic effects to that of lipid extracts from microvesicles, sphingomyelinase-treated lipid extracts lost their migration promoting activity. Further, CD147/extracellular matrix metalloprotease inducer (EMMPRIN), a plasma-membrane glycoprotein and an ECM metalloproteinase, has been demonstrated to have a crucial role in the progression of malignancies by regulating expression of VEGF and MMPs in stromal cells (Biswas et al, 1995; Tang et al, 2004).

It is interesting that in lung cancer models, hypoxia induces an increased release of microvesicles (Wysoczynski and Ratajczak, 2009). Thus, the adverse tumor microenvironment somehow triggers tumor cells to release microvesicles, which in turn facilitates angiogenesis by bringing nutrients and oxygen to the rescue of cancer cells.

Impact of MVs on tumor invasion and metastasis

Matrix degradation is essential for promoting tumor growth and metastasis (Hotary et al, 2006). As indicated above, microvesicles that are shed by tumor cells are loaded with proteases and provide an additional means of matrix degradation, creating a path of least resistance for invading tumor cells. Accordingly, several studies reported the presence of MMP2, MMP9, MT1-MMP and their zymogens urokinase-type plasminogen activator (uPA) and EMMPRIN, within tumor-derived microvesicles (Angelucci et al, 2000; Ginestra et al, 1998; Baj-Krzyworzeka et al, 2006; Hakulinen et al, 2008).

MMPs degrade basement collagens, whereas uPA catalyzes the conversion of plasminogen into plasmin, a serine protease that facilitates the conversion of MMP zymogens into their active forms as well as the degradation of matrix components such as fibrin (Angelucci et al, 2000). In addition to uPA, cathepsin B, which is also present within the microvesicles, is activated at low pH – typical of the acidic environment of solid tumors – and facilitates activation of MMPs within microvesicles (Giusti et al, 2008; Taraboletti et al, 2006).

Given the importance of matrix degradation in tumor metastases, it is logical to hypothesize that there is a direct correlation between the number of invasive microvesicles and tumor progression. Indeed, protease-loaded membrane vesicles with invasive properties have been observed in malignant ovarian ascites that are derived from women with stage-I to -IV ovarian cancer (Graves et al, 2004). This study also showed that late-stage ascites contained substantially more vesicles than those in early-stage disease, although the invasive ability of the vesicles was approximately the same, irrespective of disease stage. Similarly, in breast cancer-cell lines, the number and proteolytic capacity of shed microvesicles correlate with their in vitro invasive capacity (Ginestra et al, 1998). Both inhibition of proteases and inhibition of microvesicle adhesion to the ECM abolished the ability of these microvesicles to promote tumor invasiveness, supporting the relevance of this pathway. Recent work has shown that the small GTP binding protein ARF6 localizes to protease-containing microvesicles shed from invasive tumor-cell lines. Consistent with its role in regulating tumor invasion in cell and animal models (D'Souza-Schorey and Chavrier, 2006; Hu et al, 2009; Muralidharan-Chari et al, 2009), ARF6 activation promotes microvesicle shedding, whereas dominant inhibition of ARF6 activation attenuates microvesicle shedding (Muralidharan-Chari et al, 2009). Thus, the release of invasive microvesicles might serve in part as a mechanism by which ARF6

regulates tumor invasion.

It should be noted that microvesicle-mediated ECM degradation appears to be distinct from matrix degradation by invadopodia, another type of invasive structure that is formed at the adherent surface of tumor cells and the formation of which is also linked to the activation of ARF6. In the relevant study, cortactin, a component of invadopodia, was shown to be absent from microvesicles (Muralidharan-Chari et al, 2009).

Although proteases at the surface of invadopodia might represent a mechanism for local pericellular proteolysis at the leading or invading membrane edge, microvesicle release probably promotes more distant focal proteolysis and creation of an invasion path.

NEURONAL MVs

Different neural cells release MVs into the extracellular space. Neurons and Astrocytes shed extracellular vesicles (Figure 10) wich contains FGF2 and VEGF and maybe are involved in interaction with endothelial cells, to form the BBB (Schiera et al, 2007; Proia et al, 2008). It has been demonstrated that neurons and astrocytes control the ability of brain capillary endothelial cells to form, in a three-cell-type culture system, a barrier with permeability properties resembling those of the physiological BBB. Since in this model system physical contacts among the different cell types were not allowed, it was likely that the effects discovered were not based on direct cell-to-cell contacts. So there was the possibility that either neurons and/or astrocytes produced extracellular factors responsible for the observed effects.

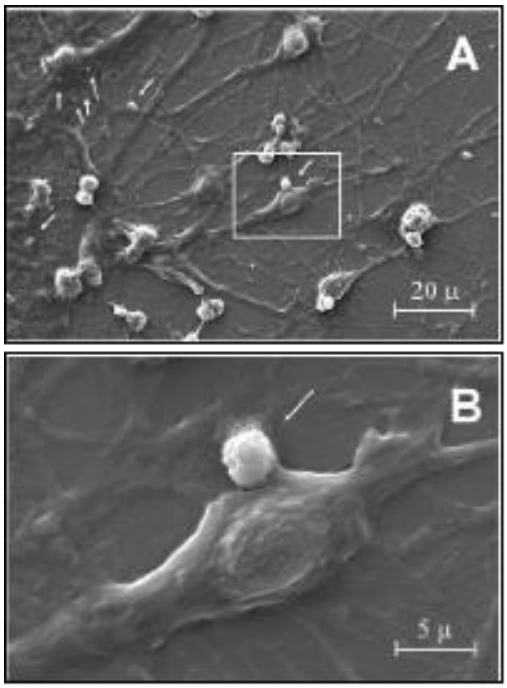

Figure 10: Analysis by scanner electron microscopy of neuronal cultures. Cells produce and release extracellular vesicular structures (indicated by arrows). A detail (in the white box) of part A was observed at higher magnification (B).

Neuronal tumoral cells, as other cells in the CNS, released microvesicles in the extracellular space. Glioblastomas release microvesicles (exosomes) containing mRNA, miRNA and angiogenic proteins. These microvesicles are taken up by

normal host cells, such as brain microvascular endothelial cells. By incorporating an mRNA for a reporter protein into these microvesicles, the message, once delivered by microvesicles, is translated by recipient cells. Microvesicles are also enriched in angiogenic proteins and stimulate tubule formation by endothelial cells. Glioblastoma microvesicles have been also shown to stimulate proliferation of a human glioma cell line, thus indicating their self-promoting properties (Skog et al, 2008).

Previous studies on oligodendroglioma cells revealed that these cells shed MVs in colture which inhibited neurite sprouting and caused apoptosis to primary fetal rat neurons, after 48-72 hours of incubation (D'Agostino et al, 2006). Proapoptotic effects of vesicles on neurons were dose-dependent and were induced also when concentrations of tumor-derived vesicles are similar to those one could imagine to occur *in vivo*, when an oligodendroglioma tumor is growing inside the central nervous system (CNS).

These vesicles contain Fas-L, a molecule that had been identified in vesicles shed by other kinds of tumor cells, and Fas receptor is reported to be expressed both in neurons and astrocytes. It was also reported that vesicle-bound Fas-L is far more active than soluble Fas-L, and that it induces apoptosis of activated T lymphocytes. Functional monoclonal antibodies against Fas-L are able to partially revert the inhibitory effects of vesicles on neurite growth and to counteract their proapoptotic effects on neurons. The presence of Fas-L in vesicles shed by tumor cells is generally considered as part of the mechanism by which tumors acquire the capability to escape from host immune surveillance. The presence of the molecule in vesicles shed by oligodendrogliomas appears to have an additional meaning in allowing production of a cell-free environment in which tumor cells can grow.

Aims Of The Project

Oligodendroglioma cells (G26/24) shed MVs in the extracellular space, as mentioned before. G26/24 vesicles are able to induce apoptosis in neurons and contain Fas-L, a pro-apoptotic factor that is not the only protein involved in the process: when neurons were treated with both MVs and inactivating anti-Fas-L antibodies, indeed, the proapoptotic effect was only partially abolished.

These data suggest that glial tumoral cells release factors that induce apoptosis in peritumoral cells. Starting from this hypothesis, my PhD project aimed at clarifying the molecular mechanisms underlying the activity of G26/24 MVs.

The project was divided into three main phases:

1. To study the ability of MVs released from oligodendroglioma cells to induce apoptosis in primary astrocytes and to clarify the mechanism involved;
2. To clarify the destiny of MVs, after shedding into the extracellular space, and the possible interactions with target cells;
3. To clarify the composition of MVs shed from oligodendroglioma cells and their possible function in the tumoral invasivness.

Materials and methods

Animals

Procedures involving animals were conducted according to the European Community Council Directive 86/609, OJL 358 1, December 12, 1987. Winstar rats (Stefano Morini, San Paolo d'Enza, Italy) were housed in the institutional animal care facility of the Department of 'Biologia Cellulare e Sviluppo', University of Palermo, Palermo, Italy, under the direction of a licensed veterinarian who approved the protocols.

Cell Culture

Astrocytes were purified from brain cortices of 2-day-old rats, as described by Cole and de Vellis (Cole and de Vellis, 1989), exploiting the differences in adhesion of the various cell types.

G26/24 oligodendroglioma cells and primary astrocytes were cultured in DME-Ham's F-12 (2:1) medium supplemented with 10% fetal calf serum (FCS), and 40 mg penicillin, 8 mg amplicillin and 90 mg of streptomycin per liter. Cell cultures were maintained in humidified 5% CO2/95% air at 37°C.

MEF (mouse embryonic fibroblast) cells are cultured in DMEM medium with 10% FCS and 40 mg penicillin, 8 mg amplicillin and 90 mg of streptomycin per liter.

PC12 cells differentiation

Undifferentiated pheochromocytoma 12 cells (PC12) were cultured in RPMI-1640 medium, containing 10% horse serum and 5% fetal bovine serum (Sigma-Aldrich). Before inducing differentiation, cells were plated on laminin. Nerve growth factor (NGF; Promega Corporation, WI, USA) was then added at an initial concentration of 4 μl/2.0x10^5 cells. The cells were then cultured for one week more. During this time cells attached to the substratum and produced a network of neurites.

Preparation of membrane vesicles from the cell culture medium

Vesicles were prepared from oligodendroglioma G26/24 subconfluent healthy cells grown in FCS-free medium. After 24 h of culture, conditioned media were centrifuged at 2.000 x g for 15 min and then at 4.000 x g for 15 min. The supernatant was centrifuged at 105.000 x g (Ti60 Rotor, Beckman) for 90 min at 4°C. Pelleted vesicles were suspended with phosphate-buffered saline pH 7.5 (PBS) and the amount of isolated vesicles was determined by measuring protein concentration by the Bradford microassay method (Bio-Rad) using bovine serum albumin (Sigma) as a standard.

Cell lysate preparation

Cultured cells were washed in PBS, scraped from the wells, and centrifuged at 1000 rpm for 5 min. The pellet was homogenized in homogenization buffer (0.32 M sucrose; 50 mM sodium phosphate buffer, pH 6.5; 50 mM KCl; 0.5 mM spermine; 0.15 mM spermidine; 2 mM EDTA; and 0.15 mM EGTA), containing the protease inhibitors aprotinin (2 μg/ml), antipain (2 μg/ml), leupeptin (2 μg/ml), pepstatin A (2 μg/ml), benzamidine (1.0 mM), and phenylmethylsulfonyl fluoride (PMSF) (1.0 mM) (all from Sigma-Aldrich, MO, USA).

SDS-PAGE

The gel is divided in “lower gel” (10-15% acrylamide) and “upper gel” (3% acrylamide) (30:0.8 acrylamide:bisacrylamide) and run in 1X running buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0,1% SDS). Polymerization is obtained by adding a catalysts APS (ammonium persulfate) and TEMED (Tetramethylethylenediamine).

Protein samples are mixed with SDS loading buffer 3X (175 mM Tris-Hcl a pH 7.5, 6% SDS W/V, 30% glicerolo, V/V, 15% β-mecaptoetanolo, 0,012% bromofenol blue) and boiled for 5 min. The gel run for about 2 h at 130 V.

Blue Comassie Staining

To identify the proteins of interest, the gel was incubated for 1 hour in Coomassie Blue staining solution (0,2% Coomassie Blue, 40% Methanol, 10% Acetic Acid), and destained in 40% Methanol, 10% Acetic Acid, for 2 hours.

Western blot analyses

After electrophoresis (SDS-PAGE), total proteins were immunoblotted on a PVDF (Polyvinylidene fluoride) membrane (Immobilon P, Millipore, MA, USA). At this aim, the gel was equilibrated with CAPS 1X (100 mM N-cyclohexyl-3-aminopropanesulfonic acid, pH 11 + 10% methanol). The PVDF membrane was activated by wetting in methanol 100% for 30 sec, and then equilibrated with CAPS 1X for 30 sec. The immunoblot was done in the "trans blot" BIORAD for 45 min at 170 mA. After blotting, the membrane, that contains the proteins, was saturated with 3% BSA, 10% BSA, PBS 1X for 1 h, and then incubated overnight with one of the following antibodies: anti-TRAIL, anti-DR4, anti-VEGF, anti-Hsp70, anti-Hsp60, anti-Hsp90, anti-Hsc70, anti-ADAMTS4, anti-ADAMTS-5, anti-BC3, anti-AGEG, anti-2-B-6, anti-GELE and anti-brevican. The antibodies are in 3% BSA, PBS 1X.

After washing with PBS containing 0,1% Tween-20, the membranes were incubated for 1 h with alkaline phosphatase-conjugated secondary antibodies. Immunocomplexes were visualized by adding the substrates of alkaline phosphatase, NBT and B-CIP (Sigma: B1911).

Co-cultures of astrocytes and G26/24 cells

Astrocytes were cultured on 6-well plates whereas G26/24 cells were plated on traslucent polycarbonate filters (transparent Transwell inserts, 23 mm diameter, 0.4 mm pore size).

The two cell types of were co-cultured for 72 hours. Finally the state of cells was tested by a vitality assay.

Treatments of astrocytes cultures with vesicles

Effects of vesicles shed from G26/24 cells into the medium (MVs) were analyzed by adding them to primary cultures of astrocytes. MVs were tested at different concentrations (3 μg to 48 μg/ml) and their effects were analyzed after 48-72 h of culture by vitality assay.

Vitality assay

Cell apoptosis was studied by staining the cells with a combination of the fluorescent DNA-binding dyes, acridine orange (AO) and ethidium bromide (EB),

100 μg/ml in PBS for each dye (AO/EB colorimetric assay). The differential uptake of these two dyes allowed the identification of viable and non-viable cells by fluorescence microscopy.
Normal nuclei in alive cells appeared bright green; apoptotic nuclei in dead cells appeared bright orange with highly condensed chromatin.

Immunofluorescence assays

Cells were fixed with 96% ethanol, on ice, for 10 min and then permeabilised by incubation with 0.1% Triton X-100 in PBS for additional 5 min. After rinsing, fixed cells were saturated by incubating them for 60 min in a wet chamber with 20% FCS and 5% bovine serum albumin in PBS. The cells were then incubated for 60 min with the following primary antibodies: rabbit polyclonal anti-VEGF (Santa-Cruz) and goat polyclonal anti-integrin ß1 (Santa-Cruz), for 60 min at RT.
After washing to remove weakly bound antibodies, cells were further incubated for 60 min with secondary antibodies, fluorescein-5-isothiocyanate (FITC) an rodamine (TRITC). We used a secondary antibody coniugated with TRITC for VEGF and a FITC-conjugated secondary antibody for integrin ß1. Nuclei were also stained with 4'-6-Diamidino-2-phenylindole (DAPI). Immunostained samples were analyzed by confocal microscopy (Olympus 1X70 with Melles Griot laser system) or by fluorescence microscopy (Olympus BX-50 microscope equipped with a Vario Cam B/W camera).

Metabolic labelling of G26/24 cells and astrocytes with ^{35}S-Methionine

G26/24 cells, astrocytes and PC12 cells, previously cultured in a methionine-containing medium, were then fed with methionine-free medium, to which 0.4 μl/ml of ^{35}S-methionine (10 μCi/μl) were added/ml of medium, and cultured for 16 hrs. At the end of treatment, the ^{35}S-methionine-containing medium was washed away and G26/24 cells were cultured for additional 24h in serum-free unlabelled medium. Conditioned medium was finally collected and centrifuged to obtain labelled vesicles. When the last conditioned medium was collected, cells were also lysed to obtain total labelled cell proteins. From astrocytes and PC12 cells total labelled cell lysates were directly prepared.
Radioactivity quantification was carried out by liquid scintillation. 1 μl of labeled proteins were precipitated with 20% (v/v, final concentration) Trichloroacetic acid, after adding 50 μg Bovin Serum Albumine, on ice for 30 min. The proteins

were pelletted by centrifugation at 15.700 x g for 15 min, washed with 200 µl of acetone and centrifuged again. Finally, proteins were resuspended in 20 µl of PBS 1X, spotted on a filter paper disk and air dried. The disks were put into propilene tubes containing 2 ml of scintillation liquid and the radioactivity was mesured.
Labelled proteins were then analyzed by western blotting. After immuno-detection membranes were also analyzed by fluorography.

Treatments of astrocytes with ^{35}S-labeled vesicles

Primary cultures of astrocytes were incubated for 24 h with ^{35}S-labeled vesicles from G26/24, and then washed with PBS. Cells were lysed to obtain total cell proteins. Total proteins from control labelled astrocytes, from astrocytes pre-incubated with labeled MVs shed by G26/24 cells, and from labeled MVs were analyzed by SDS-PAGE and fluorografy.

Co-culture of labelled G26/24 and differentiated PC12 cells

G26/24 cells were plated on traslucent polycarbonate filters and labelled with ^{35}S-methionine, in an empty well. After labelling, the filters were transferred into a well in which PC12 cells had been cultured and induced to differentiate with NGF. After 24 hours of co-culture, PC12 cells were harvested and homogenized to obtain total cell lysates. Prooteins were analyzed by SDS-PAGE and fluorografy and compared to labeled G26/24 total cell lysates and vesicles.

Aggrecanase assay

Aggrecan (500 nM) was incubated with ADAMTS5-2 (Gendron et al, 2007), ADAMTS4-2 (Fushimi et al, 2008), or G26/24 MVs in aggrecanase reaction buffer, TNCB, (50mM Tris-HCl, pH 7.5, 100mM NaCl, 10mM CaCl2, 0.02% NaN3, and 0.02% Brij 35) at 37°C for the indicated periods of time. The reactions were stopped by adding an equal volume of 2X glycosaminoglycan buffer (200 mM sodium acetate; 50mM Tris-HCl, pH 6.8, 100 mM EDTA). Aggrecan was then deglycosylated by incubating with 0.01 units of chondroitinase ABC and 0.01 units of keratanase for 16–18 h at 37°C. Proteins were finally collected by precipitation with acetone (5 volumes) at -20 °C for 18 h, and centrifugation at 3.000 x g for 10 min. The pellet was dried, dissolved in 20 µl of reducing sample buffer, and analyzed by immunoblotting using 2-B-6, BC-3, AGEG or anti-GELE

antibodies, that recognise neoepitopes generated by aggrecan cleavage. Blots were scanned and quantified using ImageJ software.

MVs incubation with heparin

Vesicles prepared from G26/24 cells, grown without heparin, were incubated with 0.1/1/10/100 ug/ml of heparin or alone as a control, for 1h at 37°C. After the incubation, the vesicles were washed in 4 ml of TNCB buffer and recovered with a centrifugation at 105.000 x g for 90 min at 4°C. The vesicles were incubated with aggrecan, as in a normal aggrecan assay.

RT-PCR

ADAMTS1, ADAMTS4 and ADAMTS5 expression was evaluated by RT–PCR of total RNA, extracted from G26/24 cells and MEF cells using a QIAamp RNA Blood Mini Kit (Qiagen Ltd, Crawley, UK).

The primers for ADAMTS1 mRNA amplification were:

5'- CAGGAAGCATAAGGAAGAAG-3' (forward) and

5'-GCACAGTGCTTAGCATCATCA-3' (reverse).

For Adamts4 mRNA amplification, the following primers were used:

5'-ATGTGGGCACAGTGTGTGAT-3' (forward) and

5'-CAAGGTGAGTGCTTCGTCTG-3' (reverse).

For ADAMTS5, the primers were:

5'-GGCATCATTCATGTGACACC-3' (forward) and

5'-CGAGTACTCAGGCCCAAATG-3' (reverse).

Isolation of total CSPGs from mouse brain extracts

A total soluble proteoglycan fraction was isolated from adult mouse brain. Extraction and fractionation of proteoglycans were essentially performed according to Yamada et al (2004). Briefly, brain tissue from an animal was homogenized in 9 volumes of ice-cold homogenization buffer (0.3 M sucrose, 4 mM HEPES, 0.15 M NaCl, pH 8.0, 1 mM EDTA, 0.25 mg/ml N-ethylmaleimide, 0.4 mM phenylmethylsulfonyl fluoride). Homogenates were centrifuged at 12.000 x g for 30 min, and the supernatant then ultracentrifuged at 378.000 x g for 60 min. The resultant supernatant was applied to an anion exchange column pre-equilibrated with buffer C (50 mM Tris, 0.15 M NaCl, 0.16 Triton X-100, pH

8.0). The column was then washed sequentially with buffer C, buffer D (50 mM Tris-HCl, pH 8.0; 0.25 M NaCl,; 0.1% Triton X-100), buffer E (50 mM Tris-HCl, pH 8.0; 6 M urea; 0.25 M NaCl; 0.1% Triton X-100), and buffer F (50 mM sodium formate, pH 3.5; 6 M urea; 0.2 M NaCl; 0.1% Triton X-100). After restoring alkaline pH, proteoglycans were eluted from the column by a linear gradient of 0.2-1 M NaCl in 50 mM Tris, 0.5% CHAPS, pH 8.0. The isolated total proteoglycan fraction was characterized by SDS-PAGE in combination with enzymatic deglycosylation.

Brevican assay

Total CSPGs were incubated with ADAMTS5-2, ADAMTS4-2, G26/24 MVs or G26/24 medium in reaction buffer (50mM Tris-HCl, pH 7.5, 100mM NaCl, 10mM CaCl2, 0.02% NaN3, and 0.02% Brij 35) at 37 °C for the indicated periods of time. The reactions were stopped by adding an equal volume of 2X glycosaminoglycan buffer (200 mM sodium acetate; 50mM Tris-HCl, pH 6.8, 100 mM EDTA). Total CSPGs were then deglycosylated by incubating with 0.01 units of chondroitinase ABC for 16–18 h at 37 °C. Proteins were finally collected by precipitation with acetone (5 volumes) at -20 °C for 18 h, and centrifugation at 3000 g for 10 min. The pellet was dried, dissolved in 20 µl of reducing sample buffer, and analyzed by immunoblotting using anti-brevican antibody.

Results

MV-INDUCED APOPTOSIS

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)

Oligodendroglioma G26/24 cells are already known to release into the colture medium MVs, which are able to induce apoptosis in primary neurons (D'Agostino et al, 2006). Investigation of the composition of MVs had already evidenced the presence of Fas-L, a proaopoptotic factor. However, inhibition of Fas-L activity reduced but not eliminated the apoptotic effects on neurons. Thus, I first investigated involvement of other factors, besides Fas-L, in inducing apoptotic processes in peritumoral cells.

A likely candidate seemed Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), a protein with pro-apoptotic activity. I searched for the presence of such a factor in MVs shed from oligodendroglioma G26/24, and found that TRAIL is visible both in G26/24 cell lysates and MVs (Figure 11). Moreover, it was found in the shed vesicles, after different periods of incubation, irrespective of supplementation of the medium with fetal calf serum (FCS). This results suggested possible involvement of TRAIL, in sinergy with Fas-L, in the proapoptotic effects of vesicles on brain cells.

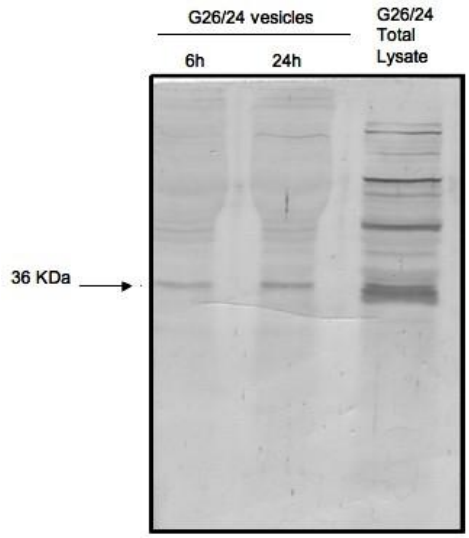

Figure 11: **G26/24 vesicles contain TRAIL.** Western blot analysis of G26/24 cell lysates and vesicles. Proteins were immunostained with an anti-TRAIL antibody.

I then looked for the presence of TRAIL receptors in brain cells. Identification of DR4 and DR5 receptors was based on western blot analysis of lysates from neurons, astrocytes, endothelial cells, G26/24 cells, PC12 cells and NIH-3T3 cells. As a positive control A375 melanoma cells were used, which are known to expose TRAIL receptors in their plasma membrane. Immunostaining evidenced signals only in the case of the DR4 receptor (Figure 12). In addition, the bands evidenced in neurons (Figure 12, lane 8) and PC12 cells (lanes 2 and 3) show a different apparent molecular mass respect to the positive control (57 KDa).

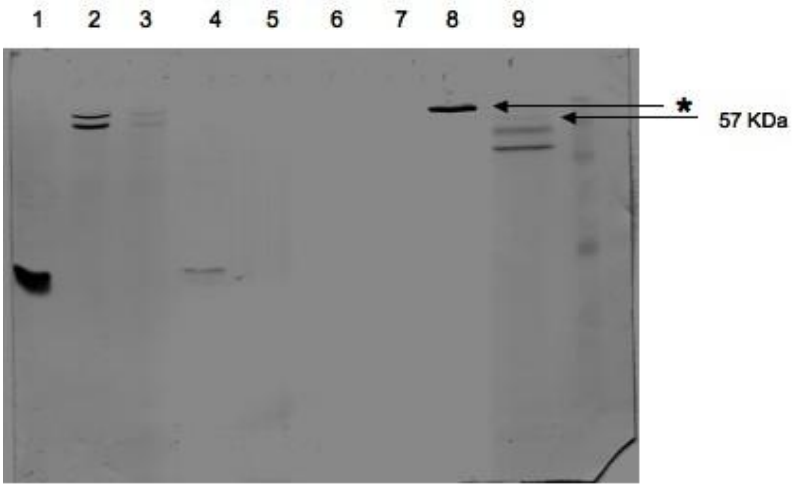

Figure 12: Immunostaining of the DR4 receptor. Western blot analysis of lysates from NIH 3T3 cells (lane 1), differentiated PC12 (lane 2), undifferentiated PC12 (lane 3), G26/24 cells (lane 4), G26/24 vesicles (lane 5), BCECs (lane 6), astrocytes (lane 7), neurons (lane 8) and A375 cells (lane 9). Proteins were immunostained with an anti-DR4 antibody.

MVs effects on primary astrocytes

To understand the role of MVs in apoptosis and their possible interaction with different brain cell types, we investigated their effects on primary astrocytes and brain capillary endothelial cells (BCECs).

The first set of experiments made use of a co-culture system in which astrocytes or BCECs were cultured with G26/24 cells. At this aim, astrocytes and BCECs were cultured in the wells of 6-well plates, and G26/24 cells were plated in special companion inserts, bearing traslucent polycarbonate filters at the bottom. As a control, we used BCECs cultured alone on the filters. After 72 hours of co-culture, total cell lysates were prepared from astrocytes and BCEC from some of the wells.

We tested, by western blot analysis, whether to be cultured with oligodendroglioma cells could induce expression of heat shock proteins in astrocytes/BCECs. We did not find any difference in the expression of molecular chaperones of the Hsc70, Hsp70 and Hsp90 classes either in astrocytes or in BCECs. The only protein affected was Hsp60, a mithocondrial heat shock protein, that seems to be more represented when astrocytes are co-cultured with other cell types, either G26/24 cells or BCECs (Figure 13).

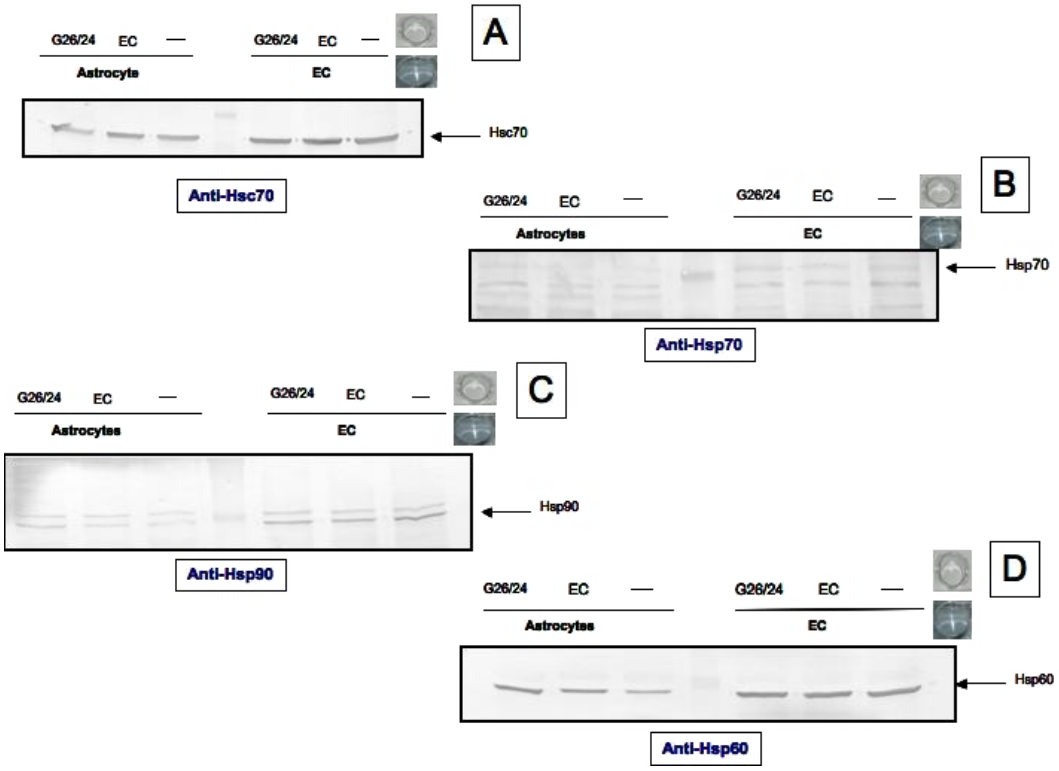

Figure 13: Western blot analysis of cell lysates from co-cultures. Western blot analysis of astrocytes and BCEC lysates after co-colturing with G26/24 cells. Proteins were immunostained with anti-Hsc70- (A), anti-Hsp70- (B), anti-Hsp90- (C) and anti-Hsp60- (D) antibodies.

In parallel experiments (i.e. co-cultured cells from some of the wells), atrocytes were used to test vitability by a mix of acridine orange and ethidium bromide (AO/EB), using as a control astrocytes cultured in the absence of G26/24 cells. These vitality assays suggested that oligodendroglioma cells did not induce apoptotic effects on astrocytes: cells cocultured for 72 hours with G26/24 cells are exactly as vital as the controls (Figure 14).

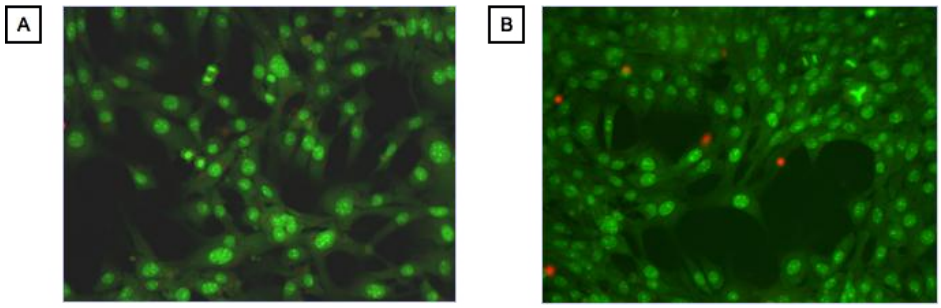

Figure 14: Effects on prymary cultures of astrocytes of co-colturing with G26/24 cells: Vitality assays with AO/EB. (A) control astrocytes; (B) astrocytes co-cultured with G26/24 cells for 72 hrs..

However, it was also possible that the number of oligodendroglioma cells in the co-culture system was too low to shed a significant amount of MVs.

Thus, we decided to directly test the effects on astrocytes of purified MVs. At this aim, G26/24 were cultured for 24 hours in a serum-free medium. At the end, the cells were pelletted, the medium was collected and the vesicles were prepared under sterile conditions.

Astrocytes were then treated for 48 hours with 40 μg of MVs and cell vitality was assayed by AO/EB staining. As shown in figure 15, many of the cells incubated with MVs died and formed apoptotic bodies. These data suggest that oligodendroglioma–shed vesicles have pro-apoptotic effects on astrocytes, even if the effects are less pronounced than in neurons.

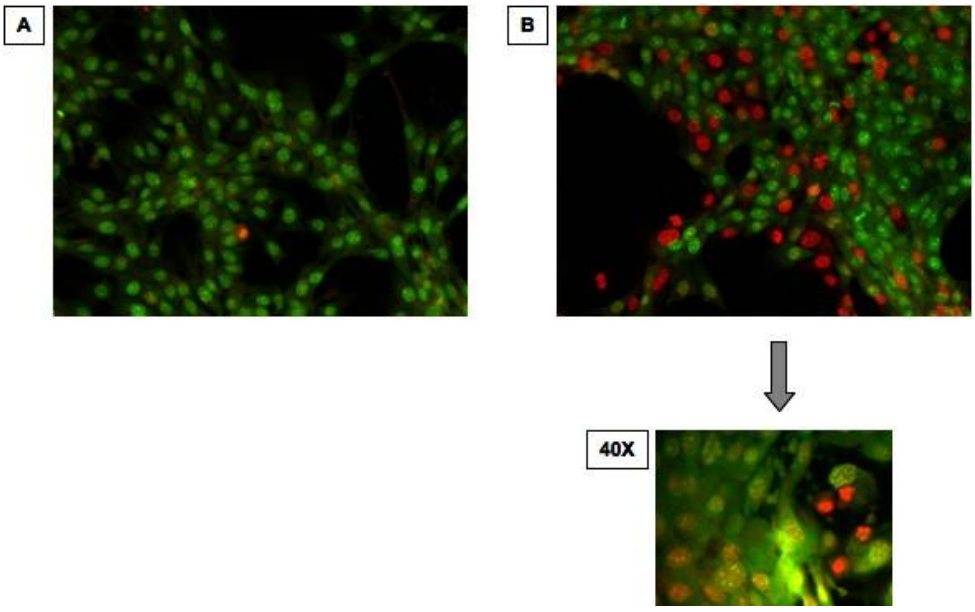

Figure 15: Effects of vesicles shed from G26/24 cells on prymary cultures of astrocytes: Vitality assays with AO/EB. (A) control astrocytes; (B) astrocytes incubated with MVs from G26/24 cells.

MVs INTERACTION WITH TARGET CELLS

Metabolic labeling of cells with ^{35}S-methionine

To clarify the fate of MVs after shedding into the extracellular space and their interaction with target cells, G26/24 cells and primary astrocytes were metabolically labeled with ^{35}S-methionine. Newly synthesized proteins contained radiolabeled methionine and, in the case of G26/24 cells, if labeled proteins entered MVs, they could be used to trace the destiny of MV components after release. Cells were incubated for 12 hours in a methionine-free medium, to make them proner to methionine uptake after this period. Then cells were grown in the ^{35}S-methionine-containing medium for 16 hours and, during this period, they started producing protein which contained labeled methionine. Cells were washed once with PBS and then coltured for 24 hours in NIH serum-free medium. From the medium in which G26/24 cells had been grown, labeled MVs were prepared. From both labeled astrocytes and G26/24 cells total cell lysates were also prepared.

Total cell lysates from both cell types and G26/24-derived MV proteins (all of which contained ^{35}S-methionine) were analyzed by SDS-PAGE and fluorography (Figure 16), to compare the labeled proteins contained in the two cell types and in MVs. This kind of analysis allowed to evidence some differences in the protein profile of total cell lysate and MVs, suggesting that some newly synthesized proteins are preferentially sorted to nascent MVs, and then transferred to the extracellular space.

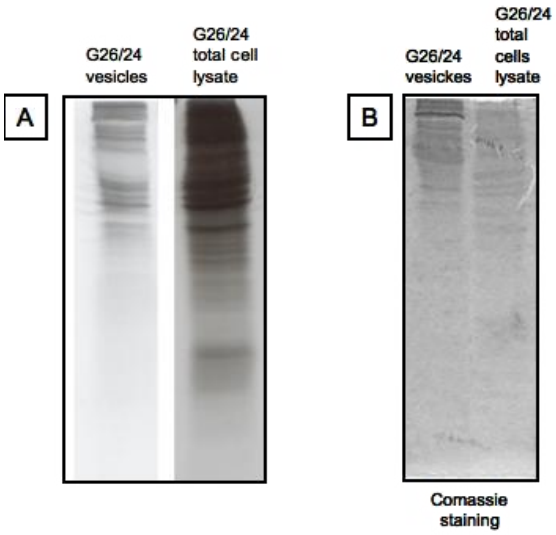

Figure 16: Metabolic labelling of G26/24 cells. Total cell lysates and MVs from G26/24 cells were analyzed by SDS-PAGE and fluorography (A) or Coomassie staining (B).

Analysis of MV target cells

In order to study the fate of MVs shed from G26/24 cells into the medium, labeled MVs were added to unlabeled target cells. In detail, astrocytes were incubated for 24 hours with about 40 μg of MVs, and then washed with PBS to eliminate the excess of labeled material before homogenizing the cells.
SDS-PAGE analysis of lysates, followed by fluorography of the gel revealed labeling of the target cells (astrocytes), suggesting trasfer of proteins to target cells through fusion of MVs with their plasma membrane (Figure 17).

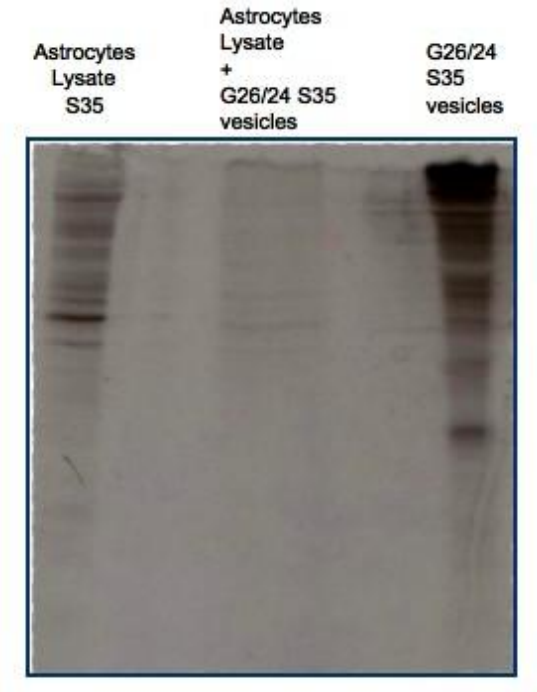

Figure 17: Fate of MVs after shedding from G26/24 cells: interaction with target astrocytes. Unlabeled astrocytes were incubated with ^{35}S-labeled vesicles shed from G26/24 cells. Total cell lysates from target astrocytes were analyzed by SDS-PAGE and fluorography.

An alternative approach was to co-culture metabolically labeled G26/24 cells with potential MV target cells.
In this case, G26/24 cells were plated in inserts bearing at bottom traslucent polycarbonate filters, placed in an empty well, and labeled with ^{35}S-methionine.After labeling, the inserts were transferred to a well in which PC12 cells had been cultured and induced to differentiate into neurons by NGF. After 24 hours of co-culture, differentiated PC12 were homogenized and the cell lysates were analyzed by SDS-PAGE and fluorografy, in comparison with labeled G26/24 total cell lysates and vesicles (Figure 18).

Figure 18: Fate of MVs after shedding from G26/24 cells: interaction with target PC12 differentiated cells. Differentaited PC12 cells were incubated with ^{35}S-labeled G26/24 cells. Total cell lysates from target cells were analyzed by SDS-PAGE and fluorography.

MVs COMPOSITION

Heat shock proteins

I also searched for the presence in MVs of Heat shock protein 70 (Hsp70). This chaperone is indeed known to be involved in the formation of both intracellular and extracellular vesicular structures, such as those related to "viral budding". Specialized membrane microdomains, known as lipid rafts, may play a role in Hsp70 exocytosis (Broquet et al 2003), inducing the realese of the factor in the extracellular space in response to cellular stress. Moreover, Hsp70 has been suggested to be involved in an exosome-dependent trafficking (Lancaster and Febbraio, 2005). Extracellular Hsp70 may act as a potent danger signal to the immune system (Vabulas et al 2002).

Starting from this observation, I tested MVs shed from G26/24 cells by western blot, with a specific anti-Hsp70 antibody. The results of these assays showed that this chaperone is highly enriched in MVs respect to the whole cell lysate, where it is not visible at all (Fig. 19b).

As a control I also studied the distribution of Hsc70 and Hsp 90. The first protein is present both in MVs and in the total cell lysate (Fig. 19a), whereas Hsp90 is only visible in the cell lysate at a very low level (Fig. 19c).

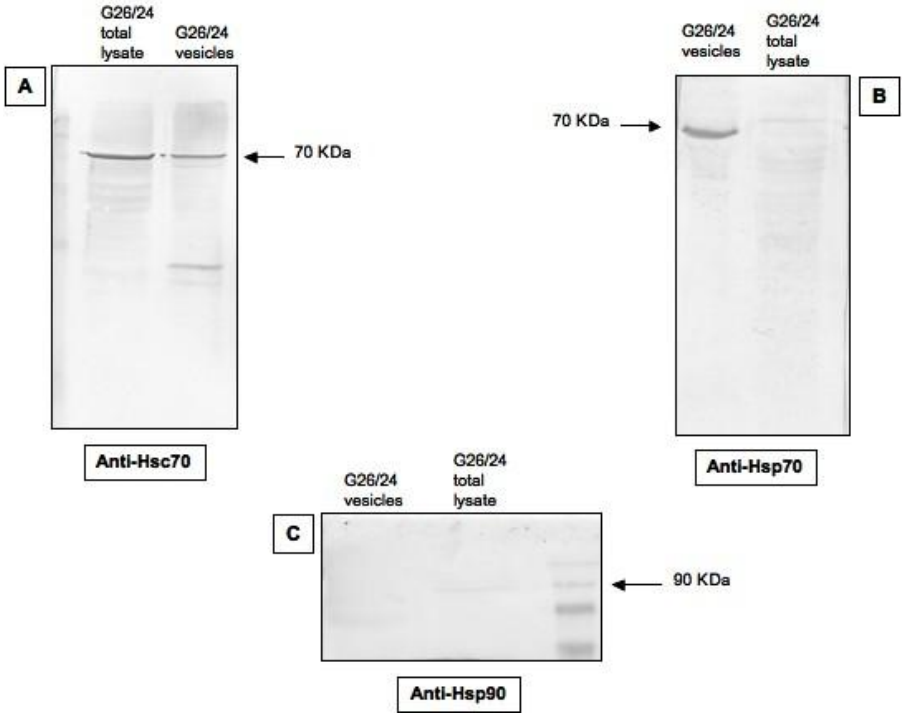

Figure 19: Vesicles shed from G26/24 cells contain Hsp proteins. Western blot analysis of G26/24 cell lysates and vesicles. Proteins were immunostained with anti-Hsc70- (A), anti-Hsp70- (B) and anti-Hsp90-antibodies (C).

Vascular endothelial growth factor (VEGF)

Among angiogenic stimuli, vascular endothelial growth factor (VEGF) is a key mediator of both physiological and pathological vessel development, particularly in tumors. VEGF is a soluble factor that acts through its tyrosine kinase receptors, mainly VEGFR-2, to directly stimulate endothelial cell functions relevant to angiogenesis, including proliferation, survival, migration, and tube formation. VEGF can also contribute to the formation of tumor neovessels by mobilizing endothelial progenitor cells from the bone marrow. Moreover, VEGF regulates vascular permeability, thus playing a role in the formation of malignant effusions. Aberrant production of VEGF has been reported in several tumor types (Ferrara et al 2003). Vesicles shed by human ovarian carcinoma cell lines CABAI and A2780 stimulated the motility and invasiveness of endothelial cells in vitro, and western blot analyses revealed the presence of VEGF in MVs (Taraboletti et al, 2006). Western analyses of both G26/24 MVs and total lysates revealed a band of about 45 KDa that could correspond to VEGF (Figure 20).

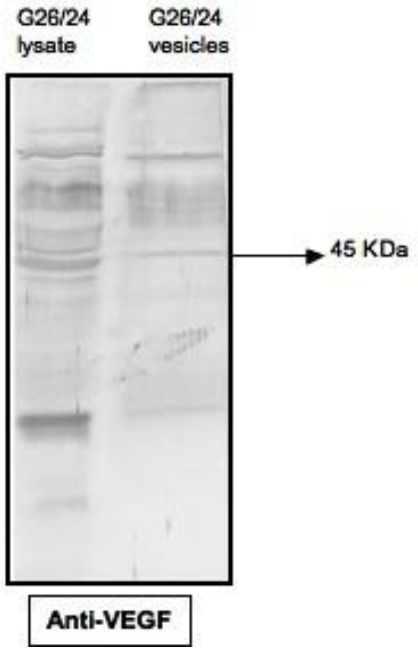

Figure 20: Vesicles shed from G26/24 cells contain VEGF. Western blot analysis of G26/24 cell lysates and vesicles. Proteins were immunostained with anti-VEGF antobody.

The presence of VEGF in MVs was also studied by double immunofluorescence, with primary anti-VEGF and anti-β1 integrin antibodies. β1 integrin was chosen

as specific marker of both the plasma membrane and extracellular shed vesicles. As shown in figure 21, vesicles bud from the membrane and contain VEGF.

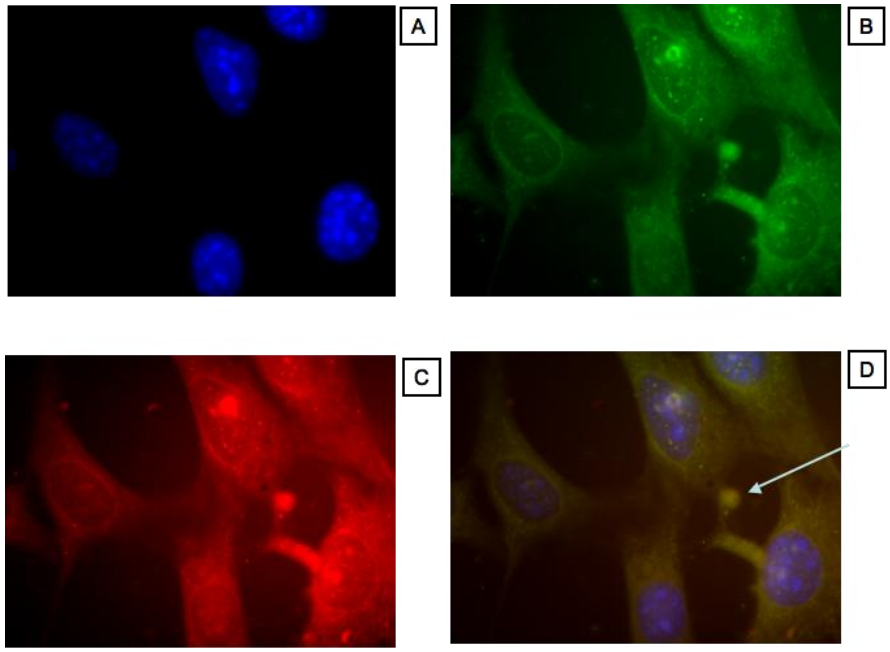

Figure 21: VEGF and β1 integrin immunofluorescence. Double immunofluorescence used to co-localize β1 integrin, in green (B) and VEGF, in red (C). Overlay of VEGF and β1 integrin is shown in D. Staining of nuclei with DAPI is shown in part A. A vesicle budding from the membrane is indicated by an arrow in part D.

POTENTIAL FUNCTIONS OF MVs IN TUMOR INVASIVNESS

Aggrecanases

Different human glioblastomas produce proteases of the ADAMTS (a disintegrin and metalloproteinase of the thrombospondin type) family, which cleave proteoglycans (Held-Feindt et al, 2006). ADAMTS-4, ADAMTS-5 and ADAMTS-1 are able to degrade aggrecan and for these reason are called aggrecanases. ADAMTSs recognize and cut, in the aggrecan molecules, specific sites, different from the ones recognized by MMPs. The aggrecanase inhibitor TIMP-3 binds the enzyme at the catalytic domain, thus blocking its activity.
I investigated the presence of aggrecanases in MVs shed from G26/24 cells and their ability to degrade aggrecan.
First of all I looked for the precence of the ADAMTS-5 and ADAMTS-4 aggrecanases in G26/24 MVs by western blot, by using specific antibodies against these factors. The western analysis did not give a good result, perhaps because of the low expression of these factors in vesicles and cells or because of the low specifity of the antibodies used (Figure 22).

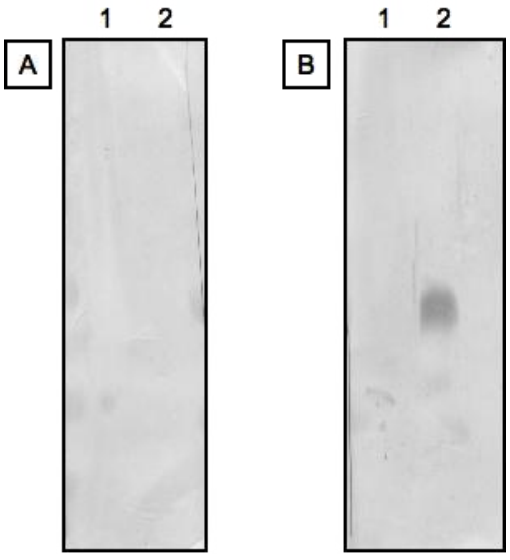

Figure 22: Analysis of aggrecanases in G26/24 MVs. Western blot analysis of MVs shed from G26/24 (1) and positive controls (2): ADAMTS-4 (A) or ADAMTS-5 (B). Proteins were immunostained with anti-ADAMTS-4- (A) or ADAMTS-5- (B) antibodies.

To be sure that G26/24 cells produce aggrecanases I tested the cells for the presence of the mRNAs encoding ADAMTS-1, ADAMTS-4 and ADAMTS-5. At

this aim, total RNA from G26/24 cells was reverse transcribed by RT-PCR with specific oligoprimers. Cultured MEF cells served as a positive expression control. As shown in figure 23, all ADAMTS mRNA were detected in G26/24 cells.

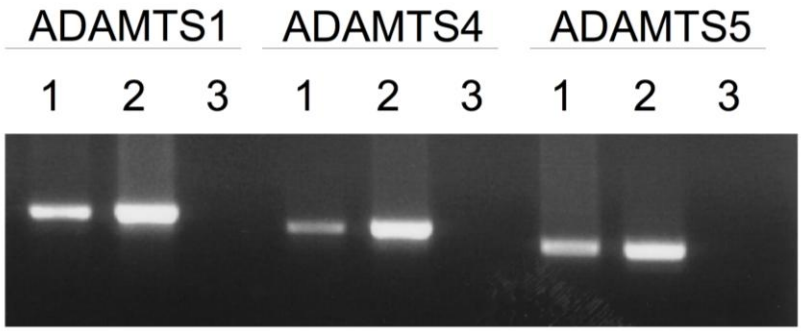

Figure 23: G26/24 cells express mRNAs encoding ADAMTS1, ADAMTS4 and ADAMTS5. RT-PCR of total RNA from G26/24 cells (lane 1) and MEF cells (lane 2). Negative controls are shown in lanes 3.

Aggrecanases activity of MVs

Because of the poor western blot results we decided to analyze directly the ability of G26/24 MVs to degrade aggrecan, by the previously described aggrecan assay (Kashiwagi et al, 2004). Nasal bovine aggrecan was incubated with enzymes, and then deglycosylated by treatment with chondroitinase ABC and keratanase. The products were analyzed by western blot, using different antibodies which recognize the following neo-epitopes, known to be generated by enzymatic cleavage of aggrecan: i) AGEG (generated by cleavage between aggrecan G2 and G3 globular domains), ii) GELE (which contain the CS2 region), iii) BC3 (which should be released by cleavage between the G1 and G2 globular domains), or the 2-B-6 antibody which recognizes chondroitin 4-sulfate stubs of the core protein, obtained after the enzymatic cleavage. A general scheme of aggregan digestion is shown in Figure 24.

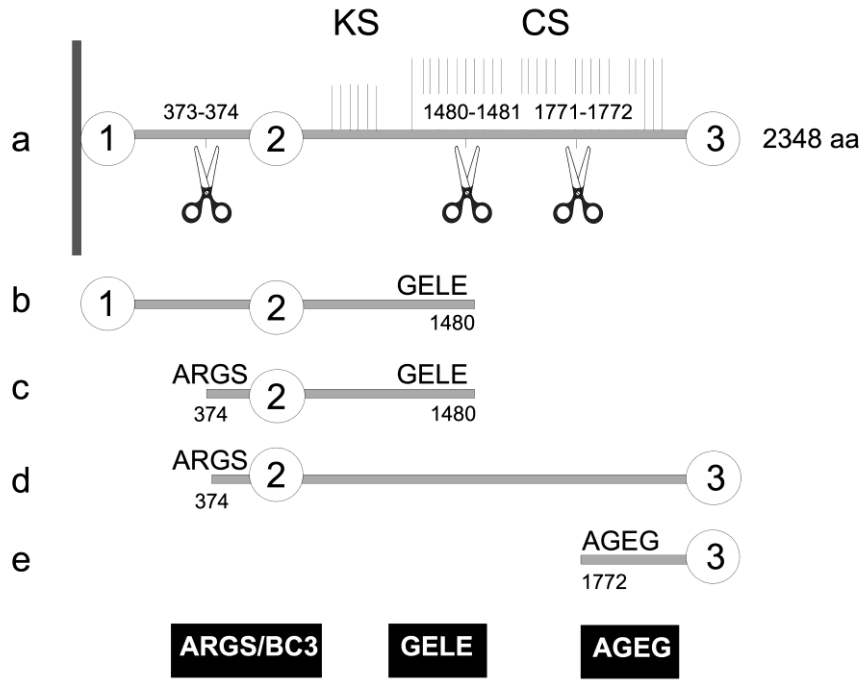

Figure 24: Schematic drawing af aggrecan core protein structure (a). Some of the sites recognized by aggrecanases (scissors) are reported, together with the fragments obtained (b-e). Neoepitope antibodies that recognize the N-terminal ARGS (BC-3 antibody) and AGEG sequences, as well as the antibody that recognizes the C-terminal GELE sequence are shown as black boxes. 1, 2 and 3: aggrecan globular domains.

Bovine aggrecan was incubated for 24 hours at 37°C with the MV fraction, recovered from the medium used to grow G26/24 cells. As a positive control, the purified ADAMTS5-2 aggrecanase (Gendron et al, 2007) was used. This assay showed that oligodendroglioma vesicles are able to cleave aggrecan at different sites, generating AGEG and GELE neoepitopes; on the other hand, they did not cut aggrecan at the site generating the neoepitope ARGS (BC3 antibody). Moreover it is also possible to obeserve the band at 100 KDa with 2-B-6 antibody, which is specific for aggrecan cleavage (Figure 25).

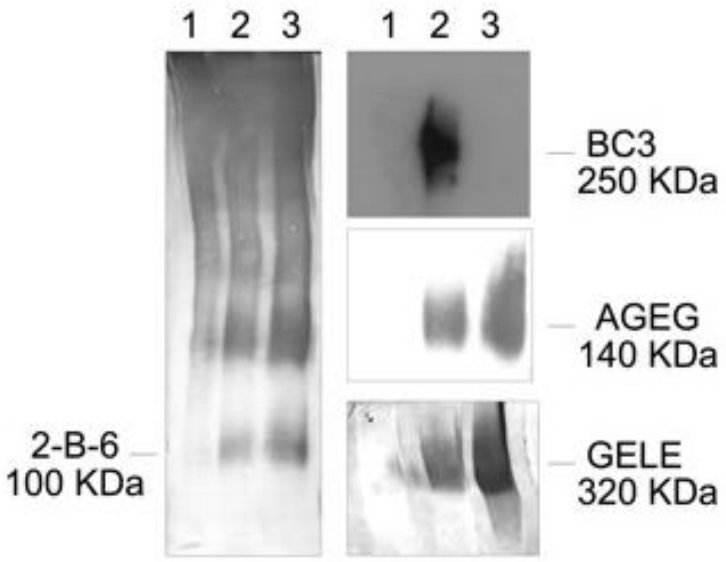

Figure 25: Vesicles shed from G26/24 cells contain aggrecan-degrading activity. Western blot analysis of aggrecan fragments released from purified bovine aggrecan (500 nM), after 24 hours of incubation at 37°C with purified ADAMTS5-2 (lane 2) or G26/24 vesicles (lane 3). Proteins were immunostained with antibodies which specifically recognize GELR, BC3, 2-B-6 and AGEG neoepitopes. As a control, aggrecan was also incubated without any enzyme (lane 1).

We also analyzed the dependence from both the incubation time and the dose of vesicles used, in the formation of neoepitope after cleavage. For these assays we tested the AGEG neoepitope formation, because this was the antibody which did the best result on western blot, both for positive control and G26/24 vesicles.

In the first assay, about 2 μg of G26/24 MVs were incubated with bovine aggrecan from 1 to 6 hours, then the samples were analyzed by western blot. AGEG neoepitope formation was already visible after one hour of incubation, increased with time and reached a plateau value after 5 hours (Figure 26).

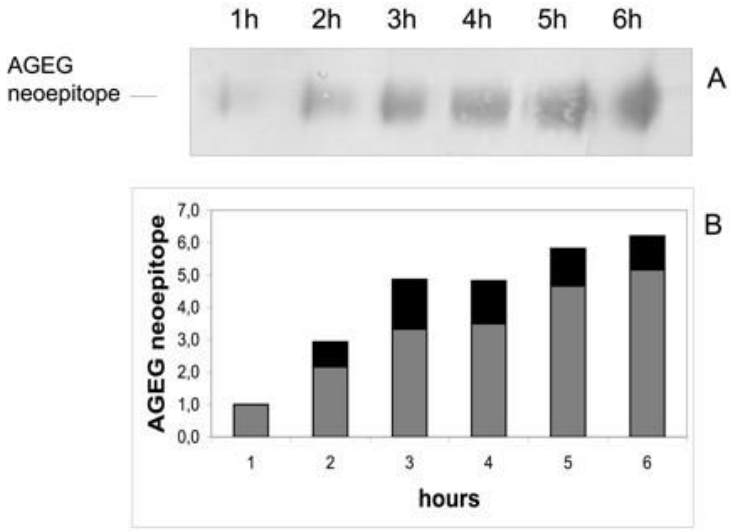

Figure 26: Aggrecan cleavage by vesicles is time-dependent. Purified bovine aggrecan (500 nM) was incubated with G26/24 vesicles at 37°C for the indicated periods of time. The reactions were stopped by adding 10 mM EDTA. Formation of the AGEG neoepitope was tested with a specific antibody.

In the dose-dependence experiment we tested the ability of G26/24 MVs to degrade more aggrecan when the quantity of vesicles is increased. Different concentrations of MVs, from 0,1 to 10 μg, were incubated with bovine aggrecan and analyzed by western blot. At 0,1 μg MVs were not able to degrade aggrecan, that start to be degaded at 0,5 μg with a peak at 2 μg (Figure 27).

In conclusion, we demonstrated that the neoepitope AGEG formation is time- and dose-dependent.

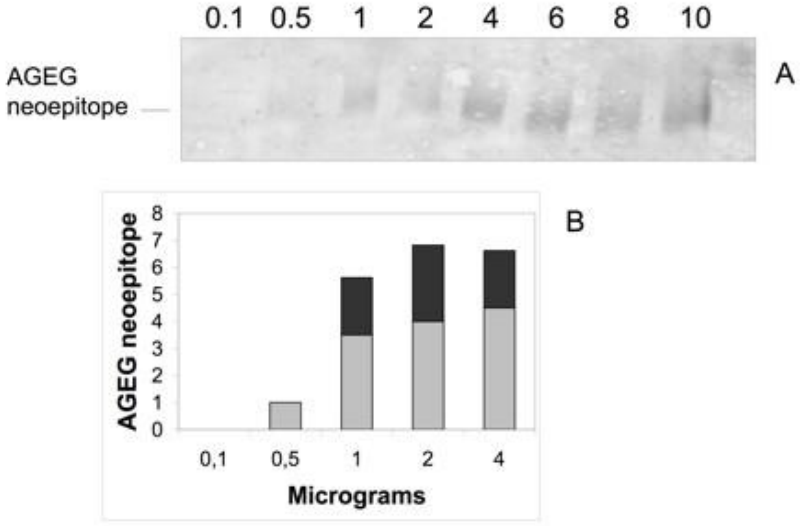

Figure 27: Aggrecan cleavage by vesicles is dose-dependent. Purified bovine aggrecan (500 nM) was incubated with different doses of G26/24 vesicles at 37°C. Formation of the AGEG neoepitope was tested with a specific antibody.

Effects of protease inhibitors on MVs aggrecan-degrading activity

The MVs enzymatic activity was then tested in the presence of specific protease inhibitors to investigate wich class(es) of proteases is (are) responsible for the cleavage. Different inhibitors were used: 10 mM EDTA, a chelator of divalent cations; 10 μM GM6001, a broad-spectrum MMPs inhibitor; 100 nM TIMP-1 and 100 nM TIMP-2, physiological inibithors of MMPs; N-TIMP-3 (5 to 100 nM), a truncated form of physilogical TIMP-3 (Lee et al, 2001).
EDTA inhibited the formation of AGEG neopepitope and 10 μM GM6001 showed only half inhibition of cleavage. TIMP-1 and TIMP-2 did not affect the enzyme activity at all, whereas N-TIMP-3 completely inhibited the cleavage, at a concentration of 100 nM (Figure 28).

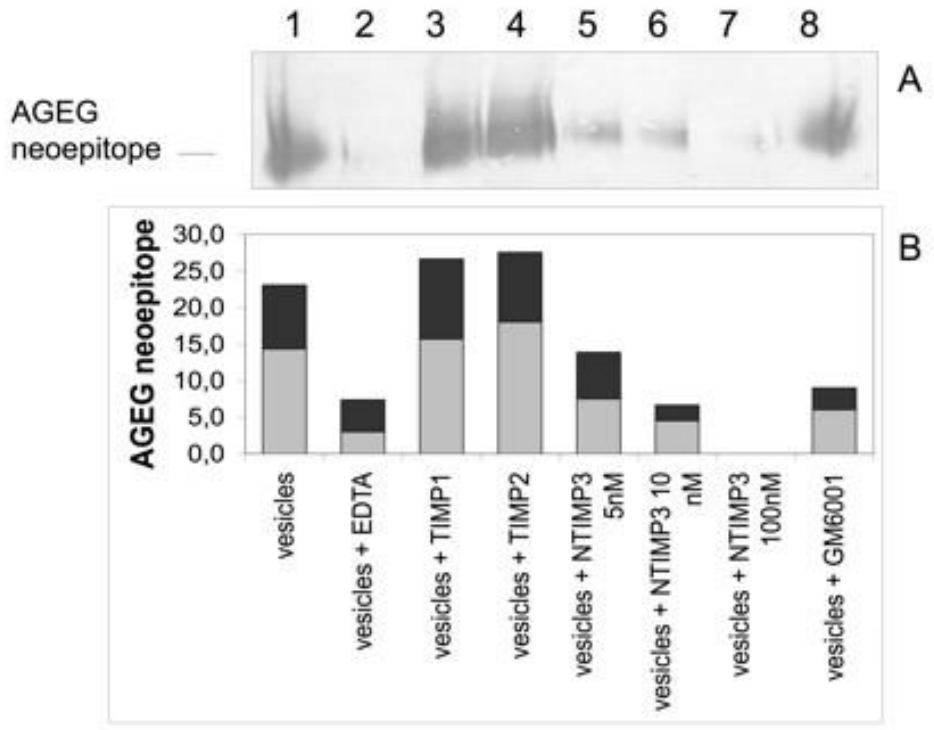

Figure 28: Aggrecan-degrading activity of vesicles is due to TIMP-3-sensitive metalloproteinases. Inhibition of aggrecanases by EDTA, TIMP-1, TIMP-2, N-TIMP-3 and GM6001. Aggrecan and G26/24 vesicles were incubated without inhibitor (lane 1) or with 10 mM EDTA (lane 2), 100 nM TIMP-1 (lane 3), 100 nM TIMP-2 (lane 4), 5 nM N-TIMP-3 (lane 5), 10 nM N-TIMP-3 (lane 6), 100 nM NTIMP-3 (lane 7) and 10 μM GM6001 (lane 8).

Increasing GM6001 concentration up to 100 μM, the enzyme anctivity was 100% inhibited (Figure 29a).
These experiments suggested that TIMP-3 sensitive aggrecanases are responsible for the cleavage of bovine aggrecan and AGEG neopepitope formation.
Aggrecan assay was repeated with ADAMTS5-2 to compare its activity in the AGEG neoepitope formation, with that observed in the case of G26/24 vesicles (Figure 29b).

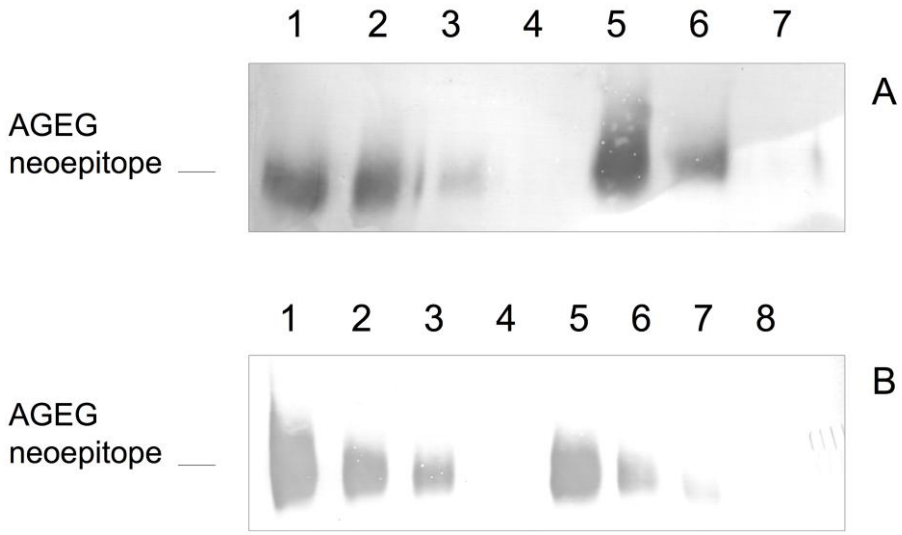

Figure 29: Inhibition of aggrecanases with different concentrations of N-TIMP-3 and GM6001. Aggrecan and G26/24 vesicles were incubated without inhibitors (lane 1), with 5 nM N-TIMP-3 (lane 2), 10 nM N-TIMP-3 (lane 3), 100 nM N-TIMP-3 (lane 4), 5 μM GM6001 (lane 5), 10 μM GM6001 (lane 6) and 100 μM GM6001 (lane 7) (A). Aggrecan was incubated with either G26/24 vesicles or ADAMTS5-2 for 3 hours without inhibitors (lane 1 and 5), with 5 nM N-TIMP-3 (lane 2 and 6), 10 nM N-TIMP-3 (lane 3 and 7), 100 nM N-TIMP-3 (lane 4 and 8).

Effects of heparin on aggrecan assay

Heparin and Calcium pentosan polysulfate (CaPPS) can inhibit aggrecan digestion by ADAMTSs and, in particular, CaPPS greatly enhances the interaction of TIMP-3 with ADAMTS-4 and -5 (Fushimi et al, 2008; Troeberg et al, 2008).
We tested the ability of heparin to dissociate the activity from the G26/24 vesicles, as this would suggest that the ADAMTSs are bound to the MVs via heparan sulfate proteoglycans.

We prepared vesicles from G26/24 cells grown without heparin, and then tested the agrecanases activity by incubating aggrecan with vesicles and 0.1/1/10/100 ug/ml of heparin.
As a control, purified ADAMTS5-2 was used (Gendron et al, 2007). Western blot results demostraded that the formation of AGEG neoepitope decreased by increasing heparin concentration, in the same way as ADAMTS5-2 (Figure 30). This could suggest that aggrecanases are indeed exposed on the external surface of MVs.

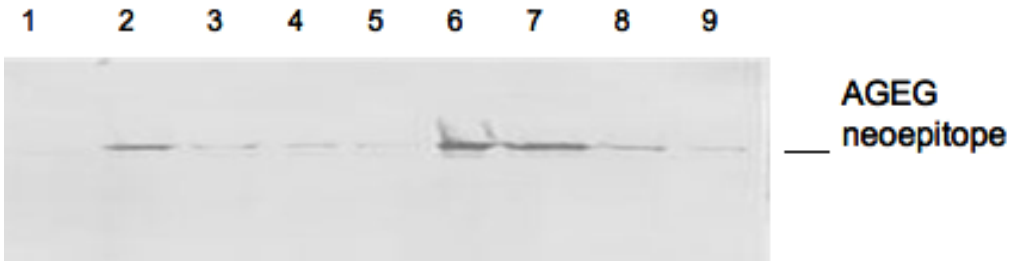

Figure 30: Aggrecan assay with heparin. Purified bovine aggrecan was incubated with ADAMTS5-2 (lanes 2-5) or G26/24 vesicles (lanes 6-9). Enzymes were incubated in the absence of heparin (lanes 2 and 6), with 1 μM heparin (lanes 3 and 7), 10 μM heparin (lanes 4 and 8) or 100 μM heparin (lanes 5 and 9). As a control, aggrecan was incubated for the same interval of time, but alone (lane 1).

To better undestand the effects of heparin on MVs, we prepared vesicles from cells grown in the absence of heparin, and incubated them with 100 ug/ml heparin for 1 h, at 37°C. Then the vesicles were diluited in TNC buffer without heparin and spinned to wash off the heparin. After this pre-incubation with heparin we tested aggrecanases activity of the washed vesicles, incubating with aggrecan (Figure 31).
The purpose of this step is to test if you can wash off the heparin and recover activity. The procedure should distinguish between the possibility that heparin inhibits activity in the vesicle (in which case washing will recover activity), and the possibility that it causes dissociation of the enzyme from the vesicle (in which case washing will not bring the activity back).

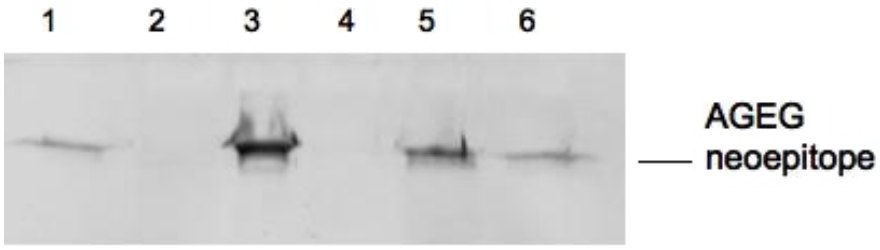

Figure 31: Aggrecan assay with G26/24 vesicles pre-incubated with heparin. Purified bovine aggrecan was incucated: with ADAMTS5-2, without heparin (lane 1) or with 100 μM heparin (lane 2); with G26/24 vesicles, without heparin (lane 3) or with G26/24 vesicles plus 100 μM heparin (lane 4); with vesicles, pre-incubated without heparin (lane 5) or pre-incubated with 100 μM heparin (lane 6).

Brevican clevage mediated by G26/24 MVs

To investigate the effects of MVs shed from G26/24 on the extracellular matrix (ECM), and to shed light on their possible role in tumor progression, the vesicle effects on brevican, another component of ECM, was also tested. Total chondroitin sulfate proteoglycans (CSPGs) fraction was purified from mouse brain, by anionic-exchange chromatography. The fraction was tested for the presence of brevican by western blot, using a specific antibody, upon treatment with chondroitinase ABC. To analyze the MV ability to cleave brevican, the purified brevican fraction was incubated with ADAMTS5-2 (Gendron et al, 2007), ADAMTS4-2 (Fushimi et al, 2008), G26/24 vesicles or G26/24 medium. ADAMTSs, used as a positive control, degrade, as expected, brevican, releasing a fragment of 55 KDa. MVs cut at different sites, including the site from which the fragment of 55 KDa originates, wich is more visible with ECL methods (Figure 32).

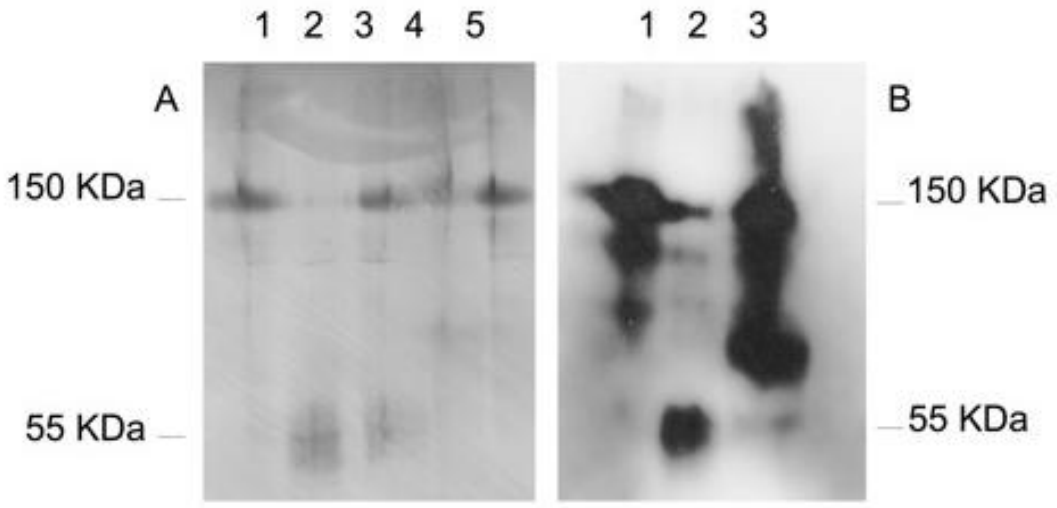

Figure 32: Cleavage of brevican by ADAMTS5 and G26/24 vesicles. Western blot analysis of brevican fragments released from brevican, after 24 hours of incubation at 37°C with purified ADAMTS5-2 (lane 2), or ADAMTS4-2 (lane 3), or G26/24 vesicles (lane 4) or G26/24 medium (lane 5). Proteins were immunostained with a specific antibody. As a control, the aggrecan was incubated without enzyme (lane 1) (A). The assay was repeated, incubating brevican alone (lane 1), with ADAMTS5 (lane 2) or G26/24 vesicles (lane 3). The membrane was developed by ECL method (B).

On the basis of these results, I concluded that vesicles shed from G26/24 cells might play a role in the proteoglycan degradation by aggrecanases, thus probably affecting ECM remodelling and hence tumor invasivness.

Discussion

Oligodendroglioma is a brain glioma tumor that develops from oligodendrocytes. All the gliomas have a remarkable tendency to infiltrate the surrounding brain and the invasive phenotype is acquired early in tumorigenesis.

Tumoral cells, as many "normal" cells, are able to commumicate with other cells or with extracellular matrix components by vesicles (MVs) that shed from the plasma membrane or are released from multivescicular bodies (MVB). These vesicles are already known to be involved in tumor progression, as in angiogenic, invasion and metastatic processes.

Several reports indicate that tumor-derived extracellular vesicles stimulate secretion of pro-angiogenic factors by stromal fibroblasts and facilitate proliferation of endothelial cells to promote angiogenesis and enable tumor growth (Al-Nedawi et al, 2009). At the same time the vesicles could contain factors that degrade the extracellular matrix, such as MMPs (Taraboletti et al, 2002).

The oligodendroglioma G26/24 cell line has been previously reported to release extracellular vesicles which inhibited neurite sprouting and caused apoptosis to primary fetal rat neurons, after 48-72 hours of incubation (D'Agostino et al, 2006). These vesicles contain Fas-L, a molecule that had been identified in vesicles shed by other kinds of tumor cells, and the Fas receptor was indeed reported to be expressed both in neurons and astrocytes (D'Agostino et al, 2006).

The present study was undertaken to better understand the mechanisms involved in vesicle-induced apoptosis as well as to identify other putative apoptosis-inducing agents. Interestingly, we found that vesicles released from oligodendroglioma cells also contain TRAIL, and that only neurons contain proteins which are immunostained by antibodies directed against TRAIL-receptors even if do not have the expected molecular mass (57 KDa). Actually, we found that astrocytes are less sensitive than neurons to the pro-apoptotic effect of MVs, maybe this is due to the specific receptors present in the cell membranes.

A second aim of this study was to shed some light on the fate of MVs after releasing into the medium. They might stay in the extracellular space very close to the site of discharge, where they could be broken down; alternatively, some vesicles might move some distance by diffusion and appear in biological fluids. In some cases microvesicles have been reported to deliver transmembrane signals

which activate surface receptors (Bianco et al, 2005); and, in still other cases, they seem to function not only as messengers, but also as platforms, necessary for the coordinate development of multisignaling processes (Del Conde et al, 2005). Possibly, MVs can receive retrograde signals and fuse with or to be taken up by their target cells. The most intriguing possibility is that these microvesicles are involved in the horizontal transfer of their membrane and/or cargo molecules, which are enriched in specific proteins and mRNAs (Deregibus et al, 2007).

On the basis of these considerations, we investigated the fate of MVs shed from oligodendroglioma cells by using a protein labelling protocol. The results obtained suggest that, at least in part, MVs fuse with targets, thus transferring their contents into the recipient cells.

The third aim of this work was to shed light on the putative role of MVs in tumor invasivness. Invasion must be viewed as a combination of the ability to migrate and the ability to modulate the extracellular space. The neural extracellular matrix has a unique molecular composition, containing only small amounts of collagens, laminin-1 and fibronectin (Ruoslahti, 1996). This matrix is primarily composed of a hyaluronic acid (HA) scaffold with associated glycoproteins and proteoglycans (PGs). The predominant group of HA-binding proteins in the neural ECM is the lectican family of chondroitin sulfate proteoglycans (CSPGs), which provides a structural and functional link between the cell membrane and the HA-based matrix scaffold (Yamaguchi, 2000). These are the most abundant CSPGs (aggrecan, neurocan, brevican and versican) in the adult CNS and have a central role as matrix organizers.

We demostrated that oligodendroglioma G26/24 membrane vesicles cleave bovine aggrecan at multiple sites, specific for aggrecanase activity, producing specific fragments, such as AGEG, GELE and ARGS (BC3 antibody).

Aggrecan cleavage by MVs was found to be time- and dose-dependent and the enzymatic activitis of MVs are inhibited by EDTA, GM6001 and N-TIMP-3, but not by TIMP-1 and TIMP-2, thus suggesting that TIMP-3 sensitive enzymes, such as aggrecanases, are involved. And indeed, a very similar cleavage and inhibition patterns were observed after incubating aggrecan with purified recombinant ADAMTS5-2, which are already known to be sensitive to N-TIMP-3 and GM6001.

This first group of analyses suggested that aggrecanases are among the putative enzymes, present in G26/24 vesicles, and able to cleave aggrecan. We also demonstrated that G26724 cells, like other glioma cells, express the mRNAs for ADAMTS1, 4 and 5.

To understand the role of the vesicles in tumor progression we also studied the G26/24 vesicles effects on brevican, a specific component of neural extracellular matrix. At this aim a protocol was set to isolate brevican from mouse brain. Purified brevican was then treated with ADAMTS4-2, ADAMTS5-2 and G26/24 vesicles. As previously reported (Matthews et al, 2000), ADAMTS4-2 and 5-2 cleave brevican at a single site. We found that also G26/24 vesicles cleave brevican at this site.

As a whole, our results suggest that MVs can be involved in ECM remodelling which accompanies and allows tumor invasivness.

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