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Recruitment of mesenchymal stem cells to apoptotic tissue cells is mediated by hepatocyte growth factor

Dissertation

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Zusammenfassung

Mesenchymale Stammzellen (MSC) besitzen geweberegenerierende und immunmodulierende Eigenschaften, verbessern das Anwachsen hämatopoetischer Stammzellen nach deren Transplantation und sind gentherapeutisch als zelluläre Vehikel einsetzbar. Ein genaues Verständnis der Rekrutierung von MSC an den Ort des krankhaften Geschehens ist zentral für ihre Funktion wie auch deren therapeutische Ausnutzung.

Der Hepatozytenwachstumsfaktor (HGF) ist ein pleiotropes Zytokin, das über seinen Rezeptor MET die Migration von MSC vermittelt. Es wird bei Gewebeschäden und auch von einigen malignen Tumoren wie dem Glioblastoma multiforme gebildet. Bei der Heilung von Gewebe hat HGF anti-apoptotische, zytoprotektive und pro-angiogenetische Effekte, im Glioblastom hingegen fördern diese Eigenschaften das invasive Wachstum des Tumors.

In der vorliegenden Dissertation wurde (i) der Einfluss von Apoptose in Abgrenzung zu Nekrose von Herz- und Nervenzellen und (ii) die Auswirkung einer Aminolävulinsäure-vermittelten photodynamischen Behandlung von Glioblastomzellen auf die Migration von MSC und die jeweilige Rolle der HGF/MET-Achse untersucht.

HL-1 Kardiomyozyten und HT-22 hippokampale Neuronen wurden apoptotisch oder nekrotisch geschädigt, U87 und U251 Glioblastomzellen wurden einer photodynamischen Behandlung unterzogen. Annexin V/Propidiumjodid- und TUNEL-Färbungen dienten dem durchflusszytometrischen Nachweis von Apoptose und Nekrose. Die chemotaktische Aktivität der Zellen für MSC wurde in *under-agarose chemotaxis assays* bestimmt. Der jeweilige Beitrag von HGF zur Migration wurde in Neutralisationsstudien evaluiert. HGF und MET wurden durch Reverse Transkription-Polymerase Kettenreaktion, *enzyme linked immunosorbent assays* und Durchflusszytometrie nachgewiesen.

Apoptotische Kardiomyozyten und neuronale Zellen induzierten die Rekrutierung von MSC, während nekrotischer Untergang der gleichen Zellen zu einem Ausbleiben dieser Migrationsantwort führte. Auch Glioblastomzellen waren chemotaktisch für MSC, und eine photodynamische Behandlung der Tumorzellen verdreifachte in etwa die Zahl einwandernder MSC. Der HGF-Rezeptor MET wurde von MSC exprimiert. HGF selbst konnte in apoptotischen, jedoch nicht in nekrotischen oder vitalen Herz- und Nervenzellen nachgewiesen werden. In Glioblastomzellen wurde eine vorhandene moderate Expression von HGF durch photodynamische Behandlung signifikant gesteigert, was mit einer Induktion von Apoptose durch die Therapie einherging. Die Neutralisation der Bioaktivität von HGF durch einen Antikörper führte zu einer signifikanten Hemmung der MSC-Migration zu allen untersuchten apoptotischen Gewebezellen.

Apoptotischer Zelltod induziert somit die Attraktion von MSC über den HGF/MET Signalweg. Auch Glioblastomzellen rekrutieren MSC über diese Achse, ein Mechanismus, der durch photodynamische Behandlung der Tumorzellen, abermals abhängig von der Induktion von Apoptose, verstärkt wird. Apoptose, nicht aber Nekrose, spielt also eine Schlüsselrolle in der Rekrutierung von MSC, was in der regenerativen Medizin wie auch in der Therapie von Glioblastomen von Relevanz sein kann.

Abkürzungsverzeichnis

ALA/PDT	*aminolaevulinic acid-mediated photodynamic therapy*
CCR	*CC chemokine receptor*
CD	*cluster of differentiation*
CXCR	*CXC chemokine receptor*
DAMP	*danger-associated molecular pattern*
DC	dendritische Zellen
GBM	Glioblastoma multiforme
HGF	Hepatozytenwachstumsfaktor
HLA	humanes Leukozytenantigen
HMGB1	*high mobility group box 1*
HSC	hämatopoetische Stammzellen
IFN	Interferon
IL	Interleukin
MET	HGF-Rezeptor
MMP	Matrix-Metalloproteinase
MSC	mesenchymale Stammzellen
SDF-1	*stromal-cell derived factor-1*
TNF	Tumornekrosefaktor
TRAIL	*tumor necrosis factor related apoptosis inducing ligand*

Inhaltsverzeichnis

1 Einleitung

1.1 Mesenchymale Stammzellen und ihre Eigenschaften

Mesenchymale Stammzellen (MSC) wurden erstmals von Friedenstein und Kollegen als stromale Vorläuferzellen im Knochenmark beschrieben [1]. Es können mittlerweile auch in zahlreichen anderen Geweben MSC identifiziert werden, etwa in Fett-, Muskel-, Periost- und Lungengewebe [2]. Sie werden typischerweise als adhärent wachsende, Fibroblasten-ähnliche Zellen gewonnen, die durch die Expression der Oberflächenmoleküle CD73, CD90 und CD105 bei gleichzeitig fehlender Expression von CD14, CD34 und CD45 charakterisiert sind [3].

MSC sind nicht-hämatopoetische, somatische Stammzellen, die zu Osteoblasten, Chondroblasten und Adipozyten, also Zellen mesenchymalen Ursprungs, differenzieren können [4]. Ob sie darüber hinaus ein keimblattüberschreitendes Differenzierungspotential besitzen [5], wie es unter anderem durch ein Auswachsen in Herzmuskelzellen [6], neuronale Zellen [7] und Hepatozyten [8] angedeutet wird, bedarf noch einer abschließenden Klärung [9]. Zweifellos besitzen MSC geweberegenerierende Aktivität, die wahrscheinlich weniger auf ihrem Differenzierungspotential, sondern vor allem auf parakrinen Mechanismen beruht [10-11].

MSC tragen ferner im Knochenmark als sogenannte mesenchymale Stromazellen zur Bildung einer Nische für hämatopoetische Stammzellen (HSC) bei [12-13]. Dieses spezifische Mikromilieu beeinflusst unter anderem über Wachstumsfaktoren und Adhäsionsmoleküle die Proliferation der HSC sowie ihren Eintritt in die Differenzierung und ihre Mobilisierung in das Blutsystem. Somit sind MSC neben regenerativen Vorgängen als Stromazellen auch an der Regulation hämatopoetischer Entwicklungsprozesse beteiligt.

Des Weiteren können MSC immunsupprimierende Aufgaben verrichten [14]. Auf der Seite der angeborenen Immunabwehr hemmen sie unter anderem die Reifung dendritischer Zellen (DC), was sich insbesondere durch eine Verminderung ihrer allostimulatorischen Potenz für Effektorzellen äußert [15]. MSC stimulieren außerdem die Produktion des immunsuppressiven Interleukin (IL)-10

durch plasmazytoide DC [16]. Da DC als spezialisierte Antigen-präsentierende Zellen eine zentrale Rolle in der Initiationsphase von T-Zell-Antworten spielen, hat die Hemmung ihrer Ausreifung, wie auch die Induktion immunsuppressiver Zytokine, Auswirkungen auf die Entwicklung der spezifischen, zellvermittelten Immunität.

Auf der Seite der adaptiven Immunität hemmen MSC die Proliferation von T-Zellen [17-18], einhergehend mit verminderter Interferon (IFN)-gamma Produktion und erhöhter IL-4 Produktion [16], was einem Shift der T-Helfer1/T-Helfer2-Polarisierung von einem pro-inflammatorischen hin zu einem anti-inflammatorischen T-Zell-Status gleichkommt. Auch konnte gezeigt werden, dass die Aktivität CD8-positiver zytotoxischer T-Zellen durch MSC gehemmt wird [19]. Dabei scheinen die Interaktionen zwischen MSC und T-Zellen unabhängig vom HLA-Typ zu erfolgen; sie werden entweder über direkten Zellkontakt [20] oder über lösliche Faktoren wie *transforming growth factor beta 1* und Hepatozytenwachstumsfaktor (HGF) vermittelt [18].

1.2 Klinische Anwendungen von mesenchymalen Stammzellen

Aufgrund ihres Differenzierungspotentials, der geweberegenerierenden Eigenschaften, der Stromafunktion und der vielfältigen immunmodulatorischen Kompetenz, kommen MSC für eine ganze Reihe an klinischen Anwendungen in Frage, insbesondere in der regenerativen Medizin, der Therapie von Autoimmunerkrankungen, hämatologischen Erkrankungen und malignen Prozessen.

Geweberegenerierende Effekte von MSC konnten am Menschen unter anderem bei Knochendefekten [21], kardialer Dysfunktion [22], insbesondere bei Patienten mit akutem Myokardinfarkt nach intrakoronarer Injektion autologer MSC aus dem Knochenmark [23-24], als auch bei zerebralem Insult [25] nachgewiesen werden. Im Rahmen der Transplantation allogener HSC bei hämatologischen Neoplasien wurden MSC zur Reduktion der Schwere einer *graft-versus host-disease* [26-27] und des Risikos einer Transplantatabstoßung [28] erfolgreich eingesetzt. Auch bei Multipler Sklerose scheint ihre immunsupprimierende Aktivität therapeutisch vielversprechend. Eine durch ein Proteolipid-Protein in der Maus verursachte autoimmune Enzephalomyelitis (ein Tiermodell für die Multiple Sklerose) konnte durch eine MSC-Applikation in ihrer

Schwere gemildert werden, was auf eine Hemmung der T-Zell-Antwort gegen das PLP-Peptid und eine verminderte Produktion spezifischer Antikörper zurückgeführt wurde [29].

MSC als zelluläre Vektoren, insbesondere für anti-tumoral wirksame Substanzen, stellen in der Gentherapie oder onkolytischen Behandlung eine attraktive Alternative zur direkten Verwendung von Viren dar, da sie aufgrund ihres Tropismus für zahlreiche Tumoren das Therapeutikum mit hoher Spezifität zum Tumor transportieren können [30]. Es konnte gezeigt werden, dass genmodifizierte, IFN-beta-exprimierende MSC *in vitro* die Proliferation von Mammakarzinom und Melanomzellen in Ko-Kulturen hemmen, was sich im Mausmodell durch die Hemmung des Wachstums der entsprechenden Tumoren bestätigen ließ [31]. Beim Glioblastoma multiforme (GBM), dem aggressivsten und häufigsten malignen Hirntumor [32], gewinnt die zelluläre Gentherapie als alternative Therapieoption eine besondere Bedeutung. Das GBM ist ein astrozytärer Tumor WHO Grad 4 mit einer nach wie vor desaströsen klinischen Prognose. Nach Standardtherapieregime, bestehend aus einer Kombination von operativer Entfernung des Tumors, fraktionierter Bestrahlung und chemotherapeutischer Behandlung mit Temozolomid, einem alkylierenden Chemotherapeutikum, betragen das mediane Überleben 14,6 Monate, die 2-Jahres Überlebensrate 27,2% und die 5-Jahres Überlebensrate 10% [33]. Insbesondere die Tatsache, dass eine komplette chirurgische Resektion des Tumors aufgrund des stark invasiven Wachstums mit Infiltration ins gesunde Hirngewebe unmöglich ist [34], trägt zur schlechten Prognose bei. Die gentherapeutische Verwendung von MSC als Transportvehikel für Zytokine, Enzyme/Prodrugs, Viruspartikel oder Oberflächenantikörper erweist sich im Kampf gegen das GBM als vielversprechend. Als Beispiele für in der GBM-Gentherapie relevante Zytokine seien das anti-tumoral wirksame IFN-beta und *tumor necrosis factor related apoptosis inducing ligand* (TRAIL), ein selektiv in Tumorzellen Apoptose-induzierender Ligand, genannt [35].

1.3 Rekrutierung mesenchymaler Stammzellen

Die Migration von MSC an den Ort des krankhaften Geschehens ist eine Grundvoraussetzung für das Erfüllen ihrer Aufgaben, vergleichbar mit der Ein-

wanderung von Immunzellen in entzündetes Gewebe. Die Migration von Zellen ist ein komplexer Prozess, an dem die Ausbildung von Lamellipodien und Filopodien, das Entstehen neuer Adhärenzkontakte, eine durch Kontraktionen im Zytoskelett hervorgerufene Translokation des Zellkörpers in die zu migrierende Richtung und die Lösung der Adhärenz am hinteren Zellpol beteiligt sind [36]. Hierbei muss unterschieden werden zwischen Chemotaxis, also gerichteter Migration entlang von Konzentrationsgradienten chemotaktisch wirksamer Substanzen, welche durch die Verteilung der jeweiligen Rezeptoren auf der Zelloberfläche der migrierenden Zelle erkannt werden, und Chemokinese, also ungerichtete Migration beziehungsweise Zellmotilität in zufällige Richtungen.

Gewebeschäden bewirken einen chemotaktischen Migrationsreiz für MSC [37]. In diesem Zusammenhang werden am häufigsten ischämisch bedingte Schäden in der Literatur beschrieben, beispielsweise am Herzen in Form eines akuten Myokardinfarkts [38-39] oder im Hirn in Form eines zerebralen Insults [25, 40].

Auch maligne Tumoren stellen einen chemotaktischen Migrationsreiz für MSC dar [30]. Die Rekrutierung von MSC in den Tumor darf verglichen werden mit dem Tropismus für Gewebeschäden. Ein maligner Prozess lässt sich als eine „Wunde, die nicht heilt“ [41] beschreiben, die, ähnlich wie ischämisch bedingte Gewebeschäden, entzündungsrelevante Mediatoren und Wachstumsfaktoren ausschüttet, von denen einige nicht nur an der Rekrutierung von Immunzellen beteiligt sind, sondern auch an der Migration von MSC [30]. Speziell bei der zellulären Behandlung von malignen Hirntumoren wie dem GBM ergibt sich durch den ausgeprägten Tumor-Tropismus der MSC, bei dem die Bluthirnschranke kein Hindernis darstellt, die Möglichkeit, MSC als Transportvehikel für anti-tumoral wirksame Substanzen [42] oder ihre direkte zytotoxische Wirkung auf maligne Zellen [43] therapeutisch auszunutzen. Dabei kann die Migration von MSC hin zum GBM durch eine zusätzliche Radiotherapie des Tumors mit Gamma-Strahlen verstärkt und somit optimiert werden [44].

1.4 Molekulare Mechanismen der Attraktion mesenchymaler Stammzellen

Die molekularen Mechanismen, die eine Rolle bei der Rekrutierung von MSC spielen, sind vielfältig und nur teilweise verstanden. Es gibt viele verschiedene Mediatoren, die in die Vermittlung von Migrationssignalen für MSC involviert sind. Dies sind vor allem Wachstumsfaktoren wie *platelet-derived growth factor*, *insulin-like growth factor* und HGF sowie eine Reihe von Chemokinen aus den CC- und CXC-Familien [37]. So wurde die Expression von zahlreichen, funktionell aktiven Chemokin-Rezeptoren auf MSC nachgewiesen: *CC chemokine receptor* (CCR)1, CCR7, CCR9, *CXC chemokine receptor* (CXCR)4, CXCR5 und CXCR6 [45]. Hieraus hervorzuheben sei CXCR4, dessen Ligand CXCL12, auch *stromal-cell derived factor-1* (SDF-1) genannt, eine zentrale Rolle vor allem für die Steuerung der Rekrutierung von HSC spielt [46-47]. In einer anderen Studie wurde neben diesen Chemokin-Rezeptoren auch die Expression von CCR2, CCR3 und CCR4 auf MSC nachgewiesen, welche durch vorherige Stimulation der Zellen mit Tumornekrosefaktor (TNF)-alpha verstärkt werden konnte, was mit einer im Vergleich zu unstimulierten MSC signifikant erhöhten Migration hin zu CCL5, CCL22 und CXCL12 einherging [48].

Die migrationsinduzierende Wirkung von HGF auf MSC [49-51], vermittelt durch den HGF-Rezeptor MET [52], ist in vielerlei Hinsicht von therapeutischem Interesse. HGF ist ein pleiotropes Zytokin, das bei Gewebeschäden vermehrt gebildet und proteolytisch aktiviert wird [50, 53], was auch bei ischämisch bedingter Herz- [54] und Hirnschädigung [55] gezeigt werden konnte. Es hat eine kardio- [56] und neuroprotektive [57] Wirkung, einhergehend mit anti-apoptotischen Eigenschaften [58-59]. HGF verstärkt auch die Proliferation und Migration von Endothelzellen, fördert die Angiogenese und trägt dadurch auch zur Wundheilung bei [60].

Im GBM wird HGF, wie auch in vielen anderen malignen Tumoren, konstitutiv gebildet [61-62]. Es ist hervorzuheben, dass HGF hier, vor allem durch seine pro-angiogenetische Wirkung, das Wachstum und die Invasion des GBM fördert [61-63] und, wie im Normalgewebe, zytoprotektiv auf die Tumorzellen wirkt [64].

1.5 HGF-vermittelter Tropismus mesenchymaler Stammzellen und Induktion von Apoptose

Apoptose, auch *programmierter Zelltod* genannt, ist ein energieabhängiger, fein regulierter Prozess, der typischerweise zu einem Absterben nicht mehr benötigter Zellen im Organismus führt [65]. Morphologisch imponieren apoptotische Zellen durch Kondensation des Zellkerns, Ausstülpung der Zellmembran und Fragmentierung der Zelle in Vesikel, auch *apoptotic bodies* genannt. Im Gegensatz zur Nekrose bleibt aufgrund einer anschließenden gezielten Phagozytose der *apoptotic bodies* durch Makrophagen in der Regel eine lokale Entzündungsreaktion aus [65]. Apoptose kann allgemein durch eine Vielzahl von Zellschädigungen, beispielsweise durch Sauerstoffmangel, Bestrahlung, Radikale, Toxine, oder auch durch einfachen Entzug von Wachstumsfaktoren verursacht werden. Zur Induktion von Apoptose kommt es entweder durch Aktivierung von Todesrezeptoren durch extrazelluläre Liganden wie Fas-Ligand, TNF-alpha oder TRAIL, oder durch Freisetzung von Cytochrom c aus den Mitochondrien ins Zytosol [66]. Letztere wird unter anderem durch anti- und proapoptotische Varianten aus der Bcl-2-Proteinfamilie reguliert und führt zu einer Aktivierung von *apoptotic protease activating factor 1*. Beide Wege führen schließlich zu einer proteolytischen Aktivierung von Adapter- und Effektorcaspasen, wodurch vor allem DNA und Zellproteine an charakteristischen Stellen gespalten werden. Auch Stress am endoplasmatischen Retikulum durch eine Anhäufung fehlgefalteter Proteine kann über einen Calcium-abhängigen Signalweg zur Apoptose führen [66].

Die Induktion von Apoptose in neuronalen Zellen führt *in vitro* zu einer erhöhten Expression von HGF [67], was eine Relevanz für den HGF/MET-vermittelten Tropismus von MSC [49-51] haben könnte. Auch die Aminolävulinsäure-vermittelte photodynamische Therapie (ALA/PDT), ein neuer Therapieansatz beim GBM [68], verursacht Apoptose in den Hirntumorzellen [69-70]. Der zytotoxische Effekt kommt dabei so zustande: Oral aufgenommenes ALA wird als Metabolit der Häm-Biosynthese zu Protoporphyrin IX umgewandelt, das sich selektiv in GBM-Zellen aufgrund einer geringen Ferrochelatase-Aktivität anreichert [71-72]. Bestrahlung mit Licht einer Wellenlänge von 635 nm führt nun zu einer Anregung von Protoporphyrin IX im Sinne einer photochemischen

Reaktion mit Bildung von reaktiven Sauerstoffspezies wie Singulett-Sauerstoff [73], welche für das Absterben der Tumorzellen verantwortlich sind [71].

Da, wie bereits angesprochen, die Produktion und Freisetzung von HGF im Zusammenhang mit Gewebeschäden [53] und malignen Prozessen wie dem GBM [61] steht, und der HGF/MET-gesteuerte Tropismus von MSC [49-51] eine therapeutische Relevanz im Hinblick auf eine Vermittlung von gewebe-regenerierenden [10] und anti-tumoralen [35, 42-43] Effekten von MSC haben kann, lag es nahe, die Bedingungen zu untersuchen, die diesen Tropismus kontrollieren. Aufgrund der bekannten anti-apoptotischen Wirkung von HGF [58-59] und MSC [74] stand bei der Untersuchung des Migrationsverhaltens von MSC die Induktion von Apoptose, in Abgrenzung zum nekrotischen Zelltod, im Mittelpunkt.

2 Ziele der Arbeit

Die Bedingungen, unter denen der HGF/MET-vermittelte Tropismus von MSC eingeleitet wird, sind zum großen Teil unklar. In „*Hepatocyte growth factor-mediated attraction of mesenchymal stem cells for apoptotic neuronal and cardiomyocytic cells*“ wurde der Einfluss von apoptotischem in Abgrenzung zu nekrotischem Zelltod auf die Rekrutierung von MSC und die jeweilige Rolle von HGF ermittelt [75]. Dies geschah vor dem Hintergrund, dass

(i) HGF bei Gewebeschäden vermehrt gebildet und proteolytisch aktiviert wird [50, 53]

(ii) HGF anti-apoptotische Eigenschaften besitzt [58]

(iii) HGF die Migration von MSC initiieren kann [49-51].

Ein besseres Verständnis der Vermittlung von geweberegenerierenden Effekten von MSC, beispielsweise beim akuten Myokardinfarkt [22] oder zerebralem Insult [25], stellte hierbei den klinischen Hintergrund dar.

In „*Migration of mesenchymal stem cells towards glioblastoma cells depends on hepatocyte-growth factor and is enhanced by aminolaevulinic acid-mediated photodynamic treatment*“ stand die Bedeutung einer Behandlung von GBM-Zellen mit ALA/PDT, einem neuen Therapiekonzept, das unter anderem zu einer Induktion von Apoptose in den Tumorzellen führt [69-70], für die Migration von MSC im Mittelpunkt - auch hier wieder unter Berücksichtigung von HGF [76]. In dieser Studie war der Hintergrund, dass

(i) HGF im GBM konstitutiv exprimiert wird [61-62]

(ii) HGF zytoprotektiv auf Tumorzellen wirkt [64], was dem Wachstum des Tumors zugute kommt

(iii) MSC zum GBM migrieren [42].

Eine mögliche verstärkte Rekrutierung von MSC durch ALA/PDT-Behandlung des GBM, vermittelt durch HGF, wurde vor dem Hintergrund der Funktion von MSC als Transportvehikel für anti-tumorale Substanzen [42] und einer möglichen direkten zytotoxischen Wirkung auf maligne Zellen [43] untersucht.

3 Originalarbeiten

3.1 Hepatocyte growth factor-mediated attraction of mesenchymal stem cells for apoptotic neuronal and cardiomyocytic cells. Vogel S, Trapp T, Börger V, Peters C, Lakbir D, Dilloo D, Sorg RV (2010) *Cellular and Molecular Life Sciences*; 67(2):295-303.

3.2 Migration of mesenchymal stem cells towards glioblastoma cells depends on hepatocyte-growth factor and is enhanced by aminolaevulinic acid-mediated photodynamic treatment. Vogel S, Peters C, Etminan N, Börger V, Schimanski A, Sabel MC, Sorg RV (2013) *Biochemical and Biophysical Research Communications*; 431(3):428-432.

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RESEARCH ARTICLE

Hepatocyte growth factor-mediated attraction of mesenchymal stem cells for apoptotic neuronal and cardiomyocytic cells

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Abstract Human bone marrow-derived mesenchymal stem cells (MSC) home to injured tissues and have regenerative capacity. In this study, we have investigated in vitro the influence of apoptotic and necrotic cell death, thus distinct types of tissue damage, on MSC migration. Concordant with an increased overall motility, MSC migrated towards apoptotic, but not vital or necrotic neuronal and cardiac cells. Hepatocyte growth factor (HGF) was expressed by the apoptotic cells only. MSC, in contrast, revealed expression of the HGF-receptor, c-Met. Blocking HGF bioactivity resulted in significant reduction of MSC migration. Moreover, recombinant HGF attracted MSC in a dose-dependent manner. Thus, apoptosis initiates chemoattraction of MSC via the HGF/c-Met axis, thereby linking tissue damage to the recruitment of cells with regenerative potential.

Keywords Mesenchymal stem cells · Apoptosis · Tissue regeneration · Chemotaxis · Cell migration · HGF · c-Met

Introduction

Human mesenchymal stem cells (MSC) are adult multipotent stem cells isolated from bone marrow (BM) and several other tissues. Typically, they are plastic adherent, non-hematopoietic cells with fibroblastoid morphology, expressing CD73, CD90, and CD105 but not the lineage markers CD14, CD34, and CD45. They are capable of differentiating into various types of mesenchymal cells, including osteoblasts, adipocytes, and chondrocytes [1, 2]. Generation of other tissue types including cardiomyocytes [3], neuronal cells [4], and hepatocytes [5] has been reported as well. The multipotency of MSC and their beneficial effects on tissue repair, which may also be due to paracrine mechanisms without extensive engraftment and transdifferentiation, make MSC a promising tool in regenerative medicine [6, 7].

In addition, MSC have stromal activity, support hematopoiesis, and may improve hematopoietic engraftment after stem cell transplantation [8, 9]. Moreover, MSC have immunosuppressive activities [7] which have already been exploited therapeutically to reduce graft-versus-host disease after allogeneic hematopoietic stem cell transplantation [10, 11].

Although local transplantation of MSC under certain conditions, like in the treatment of bone defects, may show efficacy, homing to the respective tissues is key to the therapeutic potential of MSC. Tissue injury appears to generate a strong chemoattractive signal [12]. Irradiation damage [13], ischemia of heart [14] and brain [15], chemically induced renal failure [16], and allograft rejection [17] have been reported to attract MSC. Furthermore, MSC home to various tumors, an activity which can be increased by radiotherapy and which may allow for tumor site-directed delivery of cytotoxic therapeutics using MSC as vectors [18].

The mechanisms underlying homing of MSC to the various target tissues are not entirely understood. MSC express a multitude of receptors which have been implicated in chemo-attraction, including most chemokine receptors and receptors for growth factors like platelet-derived growth factor (PDGF), insulin-like growth factor

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(IGF), and hepatocyte growth factor (HGF) [12, 18]. This variety of receptors may allow MSC to respond to distinct signals and home to different tissues [12]. However, the events generating these signals initially in the injured tissues are less well characterized.

The receptor for HGF, c-Met, is expressed on MSC [19]. HGF has been reported to be produced following tissue damage [19], including ischemia of brain [20] and heart [21], and it may become proteolytically activated in response to tissue injury [22]. It exerts neuroprotective [23] and cardioprotective activities [24] which are at least partially due to anti-apoptotic signals. However, HGF has also been shown to attract MSC [19], and a beneficial effect of MSC after myocardial infarction or stroke has been established [25, 26], implying that attraction of MSC may contribute to the tissue protective effects of HGF after ischemic injury.

In the present study, we examined whether different modes of tissue cell death, apoptosis and necrosis, are involved in initiation of MSC homing to injured tissues, and whether they differently affect MSC migration. Furthermore, we analyzed the respective contribution of HGF.

Materials and methods

Mesenchymal stem cells

Bone marrow was obtained from volunteer donors after informed consent. BM mononuclear cells (BM-MNC) were isolated by Ficoll (Biochrom, Berlin, Germany) gradient separation followed by ammonium chloride lysis of residual red blood cells. 1×10^7 BM-MNC were plated in 75-cm^2 culture flasks (Costar/Corning, Wiesbaden, Germany) and cultured at 37°C and 5% CO_2 in a humidified atmosphere in DMEM medium (Lonza, Verviers, Belgium) supplemented with 30% fetal calf serum (FCS; GIBCO/Invitrogen, Karlsruhe, Germany), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (all from Lonza). Cells were left to adhere for 48 h and then the non-adherent fraction was removed. When adherently growing MSC reached 80% confluence, they were detached with 0.25% trypsin (Lonza) and replated at 1:3. All experiments were performed with cells from passages 3–9. All MSC preparations used showed a typical [1, 2] $CD10^+$, $CD13^+$, $CD29^+$, $CD44^+$, $CD71^+$, $CD73^+$, $CD90^+$, $CD105^+$, Lin^-, and $CD45^-$ immunophenotype (data not shown). Furthermore, they differentiated along osteogenic and adipogenic pathways upon induction [1, 2] (data not shown).

Induction and detection of apoptosis and necrosis in neuronal and cardiac cells

HT-22 murine hippocampal neurons [27] were cultured in DMEM medium supplemented with 10% FCS, antibiotics, and L-glutamine. HL-1 murine cardiac myocytes [28] were cultured in Claycomb medium (Sigma–Aldrich, Taufkirchen, Germany) supplemented with 10% FCS, antibiotics, L-glutamine and 0.1 mM norepinephrine (Sigma–Aldrich). All cultures were performed at 37°C and 5% CO_2 in a humidified atmosphere.

To induce apoptosis, cells were treated with 300 nM staurosporine (Calbiochem, Bad Soden, Germany) [29] or 10 mM sodium azide (Sigma–Aldrich) for 3, 6 and 10 h or with 100 µg/ml poly (I:C) (Sigma–Aldrich) for 10 and 24 h. Necrotic cell death was initiated by incubation with 40 µM H_2O_2 (Sigma–Aldrich) for 10 h [29], 25% ethanol (Sigma–Aldrich) for 1 h, or by incubation at 56°C for 30 min. To confirm apoptosis/necrosis, annexin V/propidium iodide (PI) staining and flow cytometry were performed as recommended by the manufacturer (Beckman-Coulter, Krefeld, Germany), using a FACS Canto flow cytometer with Diva software (BD Biosciences, Heidelberg, Germany). For detection of DNA fragmentation, a terminal-deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL)-kit was used (Fluorescein In Situ Cell Death Detection Kit; Roche Applied Science, Mannheim, Germany) and data evaluated on a FACS Canto flow cytometer.

After induction of apoptosis or necrosis, cells were washed with PBS (Lonza) and incubated with fresh culture medium for 12 h to produce conditioned medium (CM). Alternatively, cells were used directly for experiments.

Scratch assay

After MSC had grown to confluence in 6-well plates (Costar/Corning), a scratch was made in the cellular layer with a sterile pipette tip over the total diameter of each well. Migration of adjoining MSC into this ‘wound’ was documented photographically after 0, 6, 12, and 24 h. Scratch assays were performed in the absence or presence of 100% CM derived from apoptotic or necrotic HT-22 and HL-1 cells.

Under-agarose chemotaxis assay

Migration of MSC towards apoptotic or necrotic brain and cardiac cells was analyzed in an under-agarose chemotaxis assay [29, 30]. 0.8% agarose (Eurogentec, Cologne, Germany) in DMEM medium was boiled, mixed with 0.5% bovine serum albumin (BSA; Roth, Karlsruhe, Germany) after cooling and poured into the wells of a 6-well plate. After the agarose had solidified, three 2-mm-wide and 5-mm-long slots 5 mm apart from each other were cut in the agarose of each well using a specifically designed stamp. Amounts of 8×10^4 MSC were then added to the central slot of each well and incubated for 2 h at 37°C and 5% CO_2

in a humidified atmosphere. During that time, left and right slots of each well were filled with 0.5% BSA/DMEM to prevent the agarose from drying-out; 70 µl of chemoattractant were then added to the left slots. CM (100%) of apoptotic and necrotic HT-22 or HL-1 cells, the cells themselves (4×10^4 cells) or 20–60 ng/ml recombinant HGF (R&D Systems, Wiesbaden, Germany) served as chemoattractants. For certain experiments, 2 µg/ml of neutralizing anti-mHGF goat polyclonal IgG antibody (AF2207; R&D Systems) or normal goat IgG (Santa Cruz, Heidelberg, Germany) were added; 0.5% BSA/DMEM in the right slot of each well served as negative control. After addition of chemoattractants, plates were incubated at 37°C and 5% CO_2 in a humidified atmosphere and migration of MSC documented after 4, 8, and 12 h. For counting migrated cells, the agarose between the center slot and the left slot was divided into 4 equally sized segments, numbered 1–4 starting at the center slot. The total number of migrated cells and the number of migrated cells in each segment subtracted by the number of cells migrating to the negative control slot/segments were documented. Only those cells were counted as migrating cells that were completely under the agarose.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was carried out with 1 µg of RNA using Transcriptor high fidelity reverse transcriptase (Roche Applied Science) and oligo-dT primers. Reverse transcription reactions without the addition of enzyme served as negative controls. RT reactions were carried out for 30 min at 50°C followed by 5 min incubation at 85°C. Resulting cDNA fragments were amplified using Taq DNA polymerase (Qiagen) according to the supplier's instructions. The cycle profile was: 3 min of denaturation at 94°C, 35 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C, followed by a final elongation step for 7 min at 72°C and cooling to 4°C. Reactions were carried out in a GeneAmp PCR System 9700 (Applied Biosystems, Darmstadt, Germany). The following forward and reverse PCR primers were used [29]: 5′-GCACTGC TTTAATAGGACACT-3′ and 5′-CCACAACCTGCATGA AGCG-3′ for human c-Met (215 bp fragment), 5′-CAT CAGCAATGCCTCCTGC-3′ and 5′-GTTCAGCTCAGGG ATGACC-3′ for human GAPDH (238 bp fragment), 5′-GTGGACAAGATTGTTATCGTG-3′ and 5′-GTGTAG TATCTCCTTCACAAC-3′ for mouse HGF (264 bp fragment) and 5′-GCAGTGGCAAAGTGGAGATTG-3′ and 5′-ATTTGCCGTGAGTGGAGTCAT-3′ for mouse GAPDH (96 bp fragment). Results were evaluated after agarose gel electrophoresis and ethidium bromide staining. HepG2 cells served as positive control for c-Met.

Detection of c-Met expression and production of HGF

Expression of c-Met on MSC was determined by flow cytometry. MSC were labeled with anti-c-Met monoclonal antibody (5 µg/ml; clone 95106, IgG1; R&D Systems) followed by FITC-conjugated F(ab)2-goat-anti-mouse IgG + M (Beckman-Coulter). Flow cytometric analysis was performed on a FACS Canto flow cytometer.

HGF levels in conditioned media derived from vital HT-22 and HL-1 cells or harvested 12 h after induction of apoptosis or necrosis (see above) were determined using an ELISA kit (Gentaur, Brussels, Belgium).

Statistical analysis

All data are presented as mean ± SEM for $n \geq 3$ unless stated otherwise. Statistical significance was determined with the Student's t test using Graph Pad Prism software (GraphPad, San Diego, CA, USA).

Results

Apoptotic but not necrotic neuronal and cardiac cells increase overall motility of MSC

To investigate the influence of apoptotic and necrotic brain and cardiac tissues on overall MSC motility, a scratch assay was used. After a scratch was made in a confluent layer of MSC, cultures were continued in the presence of CM derived from either staurosporine-induced apoptotic or H_2O_2-induced necrotic neuronal HT-22 or cardiac HL-1 cells. Recolonization of the scratch by adjoining MSC was already observed after 6 h in the presence of CM derived from apoptotic cells (data not shown), and after 24 h, MSC completely covered the scratch (Fig. 1a, c). In contrast, MSC cultured in the presence of CM derived from necrotic HT-22 (Fig. 1b) or HL-1 cells (Fig. 1d) for 24 h failed to recolonize the scratch.

MSC migrate towards apoptotic but not vital or necrotic neuronal and cardiac cells

Using an under-agarose chemotaxis assay, the specific target-directed migration of MSC was assessed. Treatment of either HT-22 or HL-1 cells with staurosporine to induce apoptosis resulted in a strong chemoattractive activity for MSC (Fig. 2a). In contrast, CM derived from vital or necrotic HT-22 and HL-1 cells obtained after treatment with H_2O_2 induced no target-directed migration of MSC

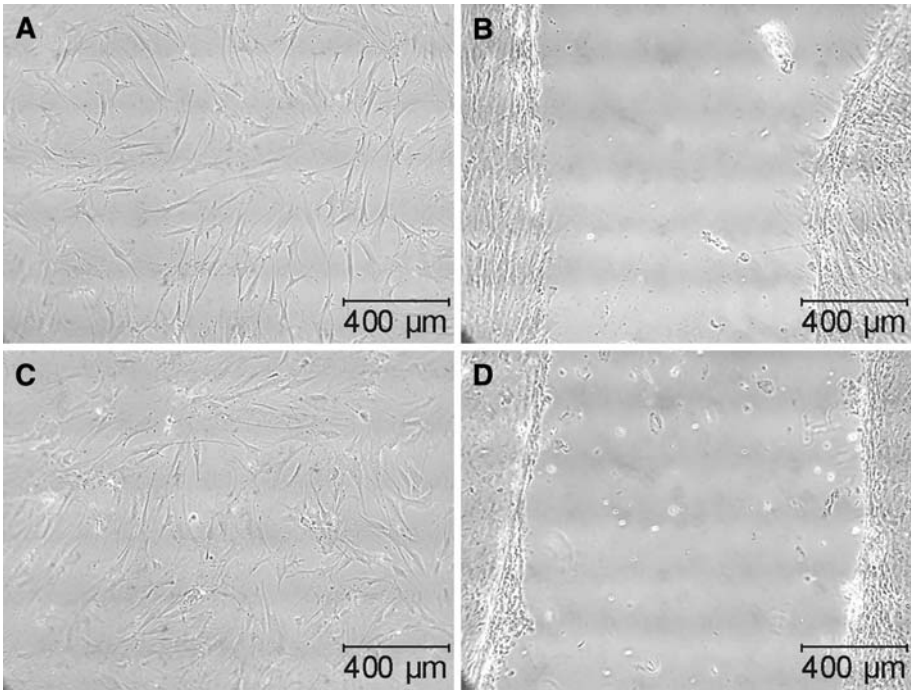

Fig. 1 Motility of MSC in response to apoptotic and necrotic cells. A scratch was made in a confluent monolayer of MSC, and cells cultured in the presence of conditioned media derived from apoptotic (300 nM staurosporine, 10 h; **a**,**c**) or necrotic (40 µM H_2O_2, 10 h; **b**,**d**) HT-22 hippocampal neurons (**a**,**b**) or HL-1 cardiac myocytes (**c**,**d**). Recolonization of the scratch by adjoining MSC was documented after 24 h

(Fig. 2a). Similar results were obtained when sodium azide or poly (I:C) were used to induce apoptosis and ethanol or incubation at 56°C to induce necrosis: only CM derived from apoptotic HT-22 and HL-1 cells constituted a chemoattractant for MSC (Fig. 2a). When the influence of the length of induction of apoptosis on the migratory response of MSC was evaluated, there was no difference between 3-, 6- or 10-h treatment with staurosporine or sodium azide (Fig. 2b) or 12- and 24-h treatment with poly (I:C) (data not shown) of HT-22 and HL-1 cells.

Total numbers of MSC migrating specifically towards the chemoattractant continuously increased over the 12-h investigation period (Fig. 2c). There was no difference whether CM or the apoptotic cells themselves were used as chemoattractant, neither in the total number of migrating cells (Fig. 2c) nor in the fraction of cells covering a certain distance within the 12-h period (Fig. 2d), indicating a comparable speed of migration.

To assess the influence of passage number on the migratory capacity of MSC, cells from passages 3, 6, and 9 were compared regarding their migration towards staurosporine-induced apoptotic HT-22 cells. There was a decrease in the total number of migrating MSC associated with increasing passage number. After 3 passages, the number of migrated MSC (1.330 ± 54 cells) was significantly higher than after 9 passages (1.010 ± 62 cells, $n = 4$; $p = 0.008$). Furthermore, the number of migrated MSC in distant agarose segments (segments 3 and 4) was higher for MSC with lower passage number. After 3 passages, 184 ± 22 MSC reached segment 4 compared to 91 ± 23 MSC ($n \geq 3$; $p = 0.049$) after 9 passages.

Extent of apoptosis and necrosis of HT-22 and HL-1 cells

To evaluate the extent of induction of apoptosis and necrosis by the various procedures, annexin V/PI and TUNEL staining were performed (Fig. 3). Induction of apoptosis in HT-22 and HL-1 cells by staurosporine or sodium azide for 10 h or by poly (I:C) for 24 h revealed Annexin V^+/PI^- early apoptotic cells as well as a smaller population ranging from 12.7 to 18.3% of Annexin V^+/PI^+ secondary necrotic cells. These secondary necrotic cells were not observed at earlier time points of induction (data not shown). At all time points analyzed, TUNEL staining detected DNA fragmentation in the majority of apoptotic cells, ranging from 90.0 to 97.3%.

Induction of necrosis by H_2O_2, ethanol or heat resulted in Annexin V^-/PI^+ necrotic cells (range 96.7–97.4%). DNA fragmentation was only detected in a minor population of the necrotic cells (range 0.3–7.1%).

The HGF/c-Met pathway mediates chemoattraction of MSC to apoptotic brain and cardiac cells

To analyze the molecular mechanism underlying MSC migration towards apoptotic brain and cardiac tissue, we determined expression of HGF in HT-22 and HL-1 cells by RT-PCR and HGF protein levels in CM derived from vital, apoptotic, and necrotic cells. Only apoptotic but not vital or necrotic HT-22 and HL-1 cells revealed HGF expression (Fig. 4a). Consistent with this observation, significant levels of HGF protein were detected only in CM of HT-22

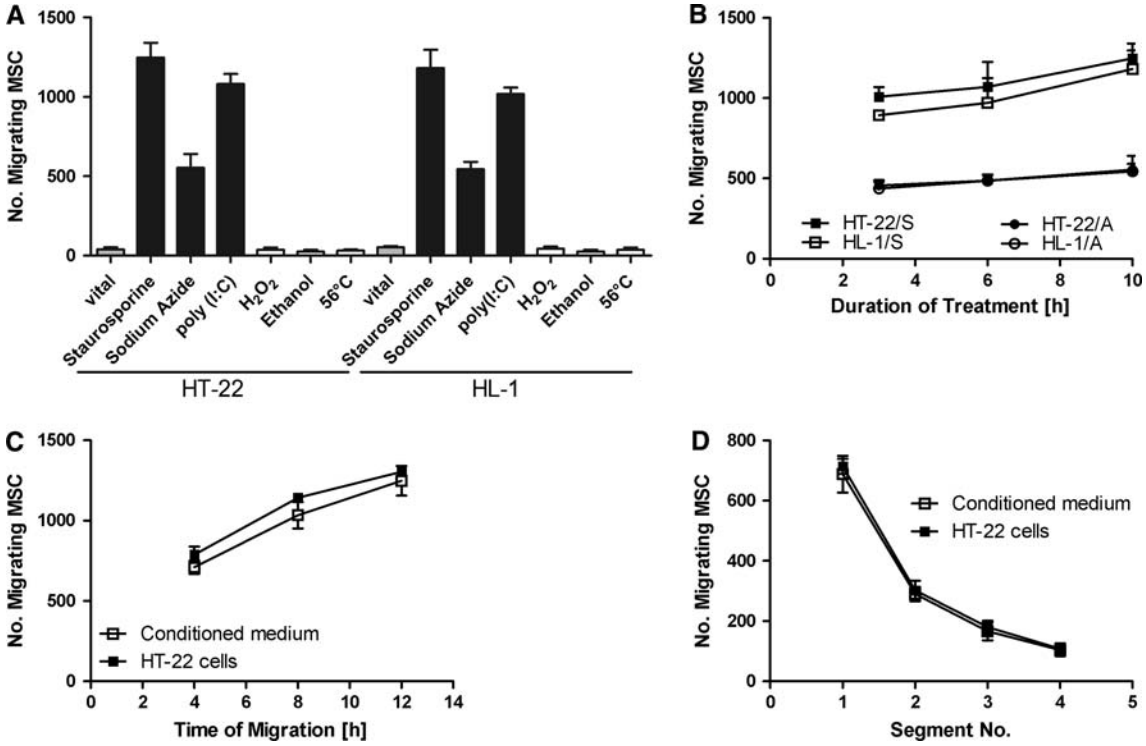

Fig. 2 Chemoattractive activity of apoptotic and necrotic neuronal and cardiac cells for MSC. Conditioned media derived from vital, apoptotic [300 nM staurosporine, 10 h; 10 mM sodium azide 10 h; 100 μg/ml poly (I:C), 24 h] or necrotic (40 μM H_2O_2, 10 h; 25% ethanol, 1 h; 56°C, 30 min) HT-22 hippocampal neurons (**a**–**d**) or HL-1 cardiac myocytes (**a**,**b**) or the cells themselves (**c**,**d**) were used as targets in an under-agarose chemotaxis assay. The total number of specifically migrating cells (**a**–**c**) and the number of cells reaching the migration segments 1–4 (**d**) were determined after 12 h (**a**,**b**,**d**) or over a 12-h period (**c**). Data are presented as mean ± SEM for $n \geq 3$. Vital, apoptotic, and necrotic cells are indicated by *grey*, *black*, and *white columns*, respectively

and HL-1 cells after induction of apoptosis with staurosporine, sodium azide, or poly (I:C), but not for vital and necrotic cells (Fig. 4b). Moreover, HGF levels only slightly increased with increasing length of apoptosis-inducing treatment (data not shown), and HGF levels measured were in good agreement with the extent of migration observed (compare Figs. 2a, 4b).

The respective receptor of HGF, c-Met, was detected in MSC by RT-PCR (Fig. 5a) as well as by flow cytometry (Fig. 5b).

Thus, there is a correlation between expression of HGF/c-Met and the migratory response of MSC to apoptotic cells. To confirm that this axis is responsible for chemoattraction of MSC to apoptotic cells, neutralization studies were performed. In the presence of a neutralizing anti-HGF polyclonal antibody, migration of MSC towards CM derived from apoptotic HT-22 and HL-1 cells was inhibited significantly ($p < 0.001$; $n \geq 3$) irrespective of whether staurosporine (78.4 ± 4.1 and 78.5 ± 5.6% inhibition, respectively), sodium azide (80.1 ± 2.6 and 80.5 ± 2.7% inhibition, respectively), or poly (I:C) (81.9 ± 2.2 and 81.7 ± 2.6% inhibition, respectively) were used to induce apoptosis (Fig. 6a). Furthermore, CM derived from apoptotic cells could be replaced by recombinant HGF. Graded doses of HGF stimulated a dose-dependent migratory response of MSC (Fig. 6b).

Discussion

MSC were shown to migrate towards apoptotic but not necrotic brain and cardiac cells in vitro. Migration correlated with c-Met expression on MSC and induction of HGF by apoptosis in the target tissues. Vital and necrotic neurons or cardiomyocytes showed no HGF expression. Moreover, blocking of HGF with a neutralizing antibody inhibited migration of MSC nearly completely. These data indicate that the HGF/c-Met axis is a key pathway involved in attracting MSC to damaged neuronal and cardiac tissues, and that tissue apoptosis constitutes a requirement to initiate these processes.

Homing of MSC to injured tissues is well established [12]. Following ischemia of heart and brain, MSC migrate to the injured tissue and contribute to tissue regeneration, although the regenerative capacity may be rather due to paracrine mechanisms than to a direct

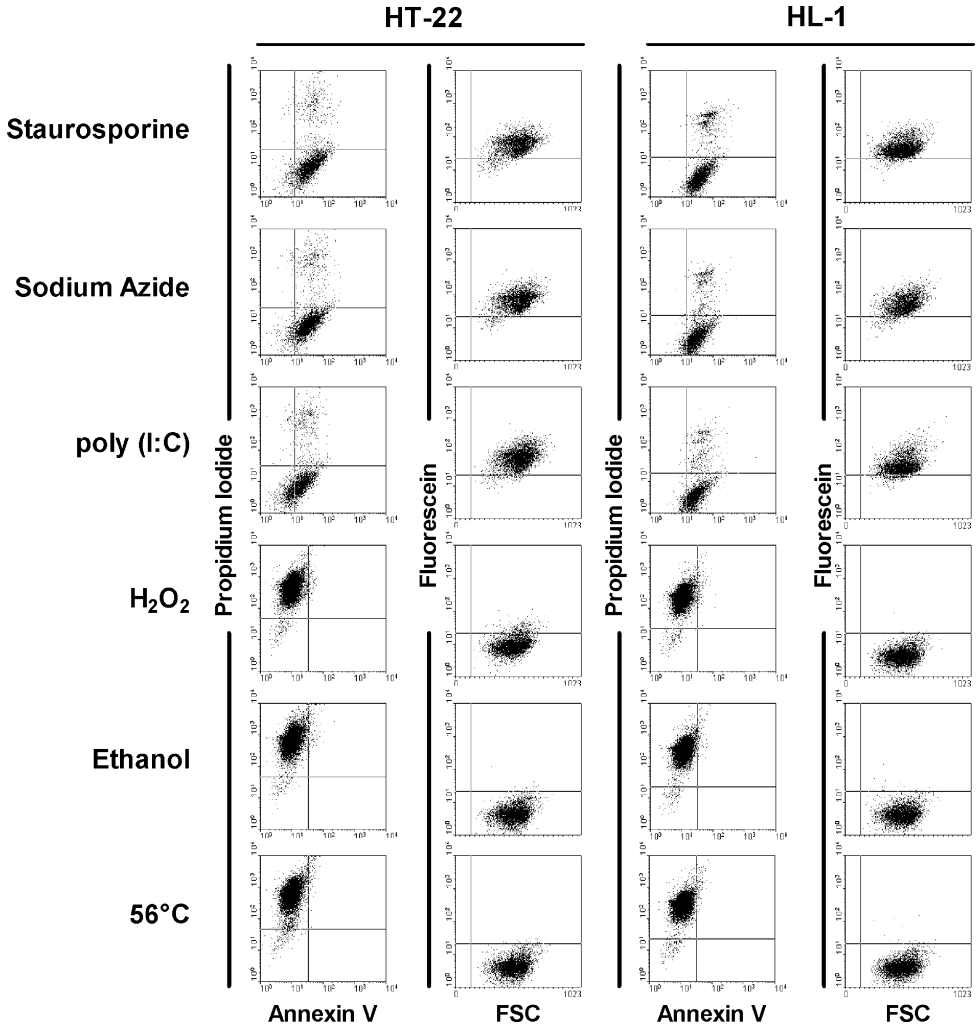

Fig. 3 Extent of apoptosis and necrosis in HT-22 and HL-1 cells. After induction of apoptosis [staurosporine, 10 h; sodium azide, 10 h; poly (I:C), 24 h] or necrosis (H_2O_2, 10 h; ethanol, 1 h; 56°C, 30 min), the frequencies of apoptotic (annexin V^+/PI^-), necrotic (annexin V^-/PI^+), and secondary necrotic cells (annexin V^+/PI^+) as well as of cells showing DNA fragmentation (fluorescein$^+$) were determined by flow cytometry. Quadrants were set according to isotype controls or to untreated cells. *FSC* Forward scatter

differentiation of MSC to the target tissue cell types [6, 14, 15, 25, 26]. HGF is produced and proteolytically activated in response to tissue injury [19, 22]. During cardiac damage, a rapid increase in HGF plasma levels has been observed [31], and cardioprotective properties of HGF have been attributed to anti-apoptotic and pro-angiogenic activities [24, 32]. Similarly, HGF production in the ischemic brain [20, 29] and neuroprotective activity of HGF due to the protection against apoptotic death of cerebral endothelial cells [33] have been reported, and this may also be true for other tissues [19, 34]. However, HGF is a potent chemo-attractant for MSC [19], and the c-Met/HGF axis is also used by other cells contributing to tissue regeneration, including endothelial progenitor cells [35], neural stem cells [36], and cord blood-derived unrestricted somatic stem cells [29]. Moreover, up-regulation of the HGF receptor c-Met on MSC, e.g., by hypoxia, is associated with increased migration towards and accelerated restoration of hind limb injury [37]. Therefore, the HGF-mediated guiding of MSC towards sites of tissue damage may also contribute to the cytoprotective activity of HGF.

Only apoptotic cells produced HGF and induced HGF-dependent migration of MSC. Necrotic cell death failed to result in HGF production and no migration of MSC towards necrotic neurons or cardiomyocytes was observed. Thus, the type of cell death, i.e., the type of tissue damage, may allow for distinct cellular response patterns to occur. Apoptosis resulted in production of HGF which on the one side has anti-apoptotic activities and on the other side attracts cells with regenerative potential, providing the basis for limiting apoptosis and initiating tissue repair.

Different cellular responses on apoptotic versus necrotic cell death have also been reported for the immune system. Necrotic cell death constitutes an immunostimulatory signal resulting in activation of dendritic cells (DC) and induction of immunity whereas apoptotic cell death appears to be associated with immunological tolerance although the microenvironment may modulate this outcome [38]. HGF [39] and MSC [7]

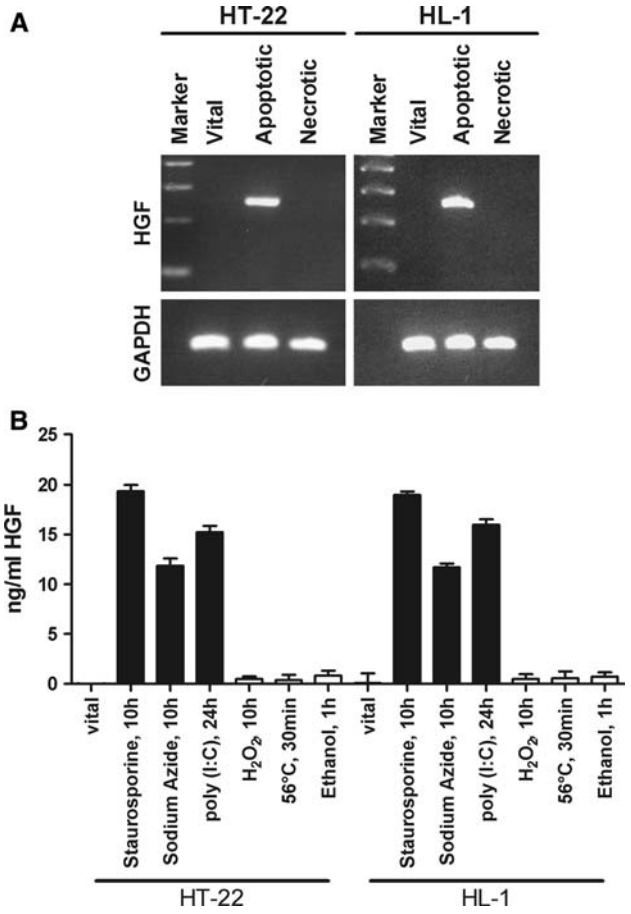

Fig. 4 Expression of HGF in HT-22 and HL-1 cells. mRNA expression of HGF in vital, apoptotic (300 nM staurosporine, 10 h) and necrotic (40 μM H_2O_2, 10 h) HT-22 hippocampal neurons and HL-1 cardiac myocytes was determined by RT-PCR (**a**). GAPDH served as positive control, reactions without reverse transcriptase (all negative, data not shown) served as negative controls. A 100-bp ladder was used as size marker. HGF levels in conditioned media derived from vital, apoptotic [300 nM staurosporine, 10 h; 10 mM sodium azide 10 h; 100 μg/ml poly (I:C), 24 h] or necrotic HT-22 and HL-1 cells (40 μM H_2O_2, 10 h; 25% ethanol, 1 h; 56°C, 30 min) were measured by ELISA (**b**). Vital, apoptotic, and necrotic cells are indicated by *grey*, *black*, and *white columns*, respectively. Data are expressed as mean ± SEM for $n \geq 2$

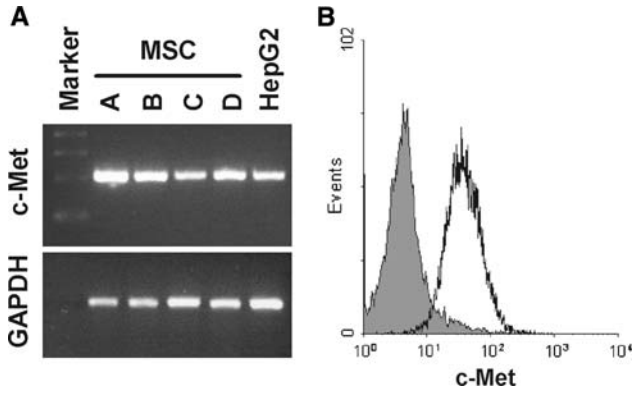

Fig. 5 Expression of the HGF receptor c-Met in MSC. Expression of c-Met in 4 MSC lines (MSC *A–D*) was detected by RT-PCR (**a**). GAPDH and RNA from the cell line HepG2 served as positive controls and reactions without reverse transcriptase (all negative, data not shown) served as negative controls. A 100-bp ladder was used as size marker. Surface expression of c-Met on MSC (**b**) was detected by staining with a c-Met specific monoclonal antibody and flow cytometry (*open histogram*). Negative control staining is indicated by a *gray* histogram

have immunosuppressive properties and affect dendritic cell as well as T-cell activation and function. Therefore, apoptosis appears not only to set off processes to limit apoptosis and to favor tissue regeneration but also immunological tolerance. In contrast, necrosis is associated with pro-inflammatory signals which generate an immunostimulatory microenvironment and result in recruitment of dendritic cells, thereby laying the path to immunity.

Interestingly, although in this study no migration towards necrotic cells was detected, Meng et al. [40] reported migration of MSC towards high mobility group box 1 (HMGB-1), a nuclear protein passively released upon necrotic cell death [41]. Whether this reflects heterogeneity of MSC, subsets of cells responding to distinct signals, or unique tissue-specific signals is currently unknown. c-Met was expressed on all cells homogenously and a negative subset was not identified. Nevertheless, contribution of other factors cannot be ruled out completely, because neutralization of HGF bioactivity inhibited migration of MSC only by about 75%. Other reports indicated the CXCL12/CXCR4 axis to be involved in homing of MSC to the injured heart [42] and brain [43]. However, blocking the CXCL12–CXCR4 interaction did not inhibit homing completely either [42], or it caused no inhibition at all [44]. Homing to tissue injuries is a complex process which may use different pathways for distinct tissues, and there may also be redundancy in chemoattractants guiding MSC to individual tissues. MSC express a multitude of chemokine and growth factor receptors involved in chemo-attraction [12, 18], some of which are expressed on subsets of cells only like CXCR4, CCR1, and CX3CR1 [45]. Other molecules including ligands of selectins may also contribute to tissue-specific homing [46]. Furthermore, culture-dependent differences in expression of receptors as well as in migration of MSC [47] have been reported, and the source of cells, e.g., bone marrow, placenta and cord blood, influences migratory potential [48]. In the present study after longer culture periods, MSC showed the same chemotaxis pattern towards apoptotic neurons and cardiomyocytes. However, a decline in migration with increasing passage number was observed.

In conclusion, we have identified a mechanism, apoptosis, which initiates migration of MSC after neuronal and

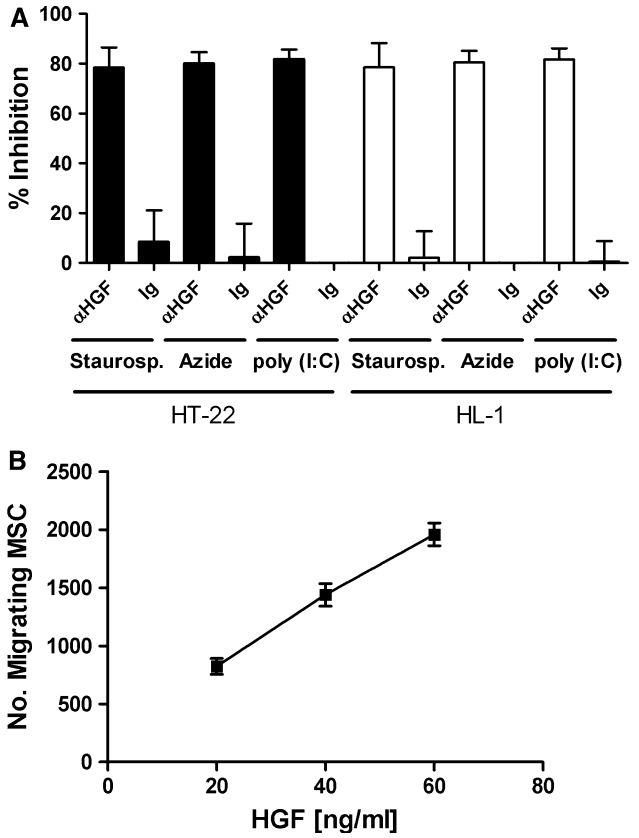

Fig. 6 Role of HGF/c-Met in MSC migration towards apoptotic neuronal and cardiac cells. Conditioned media of apoptotic HT22 hippocampal neurons (*black columns*) or HL-1 cardiac myocytes (*white columns*) treated with 300 nM staurosporine for 3 h, 10 mM sodium azide for 3 h, or 100 μg/ml poly (I:C) for 10 h with or without the addition of 2 μg/ml anti-HGF neutralizing antibody or control antibody (**a**) or graded doses of recombinant HGF (**b**) served as targets for MSC in an under-agarose chemotaxis assay. Total numbers of specifically migrating cells were counted after 12 h. Data (mean ± SEM for $n \geq 3$) are presented as % inhibition calculated from the number of migrating cells in the absence of antibodies set to 0% (**a**) or as number of migrating cells (**b**)

cardiac tissue injury and could show that the HGF/c-Met axis is the key pathway involved.

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Migration of mesenchymal stem cells towards glioblastoma cells depends on hepatocyte-growth factor and is enhanced by aminolaevulinic acid-mediated photodynamic treatment

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ABSTRACT

Hepatocyte-growth factor (HGF) is expressed by glioblastomas and contributes to their growth, migration and invasion. HGF also mediates migration of mesenchymal stem cells (MSC) to sites of apoptotic cell death. Moreover, MSC show tropism for glioblastomas, which is exploited in gene therapy to deliver the therapeutics to the tumor cells. Here, we have studied whether HGF contributes to the recruitment of MSC by glioblastoma cells and whether aminolaevulinic acid-mediated photodynamic therapy (ALA/PDT), a novel therapeutic approach that induces apoptosis in glioblastoma cells, affects HGF release and this migratory response. MSC expressed the HGF receptor MET and migrated towards U87 and U251 glioblastoma spheroids. Migration increased significantly when spheroids were subjected to ALA/PDT, which was associated with induction of apoptosis and up-regulation of HGF. Neutralizing HGF resulted in significant inhibition of MSC migration towards untreated as well as ALA/PDT-treated spheroids. Thus, glioblastoma cells express HGF, which contributes to the attraction of MSC. ALA/PDT induces apoptosis and augments HGF release causing enhanced MSC migration towards the tumor cells. ALA/PDT may therefore be exploited to improve targeting of MSC delivered gene therapy, but it may also constitute a risk in terms of beneficial effects for the tumor.

1. Introduction

Glioblastoma is the most frequent and aggressive malignant primary brain tumor [1]. Despite multimodal therapy combining surgery, radiotherapy and alkylating chemotherapy, prognosis of patients is dismal: median survival is 14.6 months, the 2-year survival rate 27.2% [2]. Aminolaevulinic acid-mediated photodynamic therapy (ALA/PDT) is a novel therapeutic approach for glioblastoma, and early clinical results are promising [3,4]. ALA is an intermediate of the heme biosynthesis pathway. Oral uptake of ALA results in preferential accumulation of protoporphyrin IX (PPIX) in glioblastoma cells, mainly due to low ferrochelatase activity [5,6]. This preferential accumulation can be exploited for intraoperative identification of the tumor during fluorescence-guided surgery using light of 400 nm wavelength for illumination, allowing to increase the extent of resection, associated with higher progression-free survival [7]. However, when exposed to light of 635 nm wavelength, PPIX acts as a potent photosensitizer [6]. Its excitation initiates a photochemical reaction, which kills the tumor cells via the generation of singlet oxygen [5]. In addition to this direct phototoxic effect, there appears to be an immunological component to ALA/PDT, and it has been shown to influence the tumor vasculature as well as the migratory and invasive behavior of tumor cells [5,8–11].

We and others have previously shown that ALA/PDT induces apoptosis of glioblastoma cells [11,12]. The type of cell death has consequences besides killing of the tumor cells, e.g. it may affect the development of immunity [13]. Furthermore, apoptosis but not necrosis of neurons has been shown to result in up-regulation of hepatocyte-growth factor (HGF) [14,15]. HGF is a pleiotropic cytokine with anti-apoptotic activity [16]. It promotes glioblastoma growth, invasiveness and angiogenesis [17–19], and its tyrosine kinase receptor MET may represent a marker for mesenchymal and proneural glioblastoma stem cell subtypes [20]. Therefore, the HGF–MET pathway is currently evaluated as

Abbreviations: ALA/PDT, aminolaevulinic acid-mediated photodynamic therapy/treatment; HGF, hepatocyte-growth factor; MSC, mesenchymal stem cell(s).

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a potential therapeutic target in glioblastoma [21]. On the other hand, HGF is a chemoattractant for mesenchymal stem cells (MSC) [14,22,23]. MSC are multipotent stem cells found in bone marrow and other tissues. They are capable of differentiating into various types of mesenchymal cells, including osteoblasts, adipocytes and chondrocytes [24]. It is well established that MSC home to glioblastomas and this tropism is utilized to target therapeutics to the tumors, including cytokines, enzymes/pro-drugs, oncolytic viruses, toxins and others [25].

Expression of HGF by glioblastoma cells [17,19] and its activity as a chemotactic stimulus for MSC [14,22,23] as well as the observation that apoptosis, thus, the type of cell death, which is induced by ALA/PDT [11,12], is associated with induction of HGF in neurons [14,15], raises the questions whether HGF is involved in the migration of MSC towards glioblastoma cells and whether ALA/PDT-induced apoptosis results in increased HGF release by glioblastoma cells and an enhanced migratory response of MSC.

2. Materials and methods

2.1. Mesenchymal stem cells

Human bone marrow was obtained from volunteer donors after informed consent with the ethical approval of the local ethical committee. MSC were isolated from bone marrow as described previously [14]. Briefly, bone marrow cells were plated in 75-cm^2 culture flasks (Greiner, Nürtingen, Germany) and cultured at 37 °C and 5% CO_2 in a humidified atmosphere in DMEM (Lonza, Verviers, Belgium) supplemented with 30% fetal calf serum (FCS; GIBCO/Invitrogen, Karlsruhe, Germany), 50 μg/ml gentamycin and 2 mM L-glutamine (all from Lonza). After 48 h, non-adherent cells were removed and cultures continued. When reaching 80% confluence, cells were harvested with trypsin (Lonza) and re-plated at 1:3. All MSC preparations showed the immunophenotype, and osteogenic and adipogenic differentiation typical of MSC [24].

2.2. Glioblastoma spheroids and ALA/PDT

Culture and generation of spheroids as well as ALA/PDT of U87 and U251 glioblastoma cell lines were performed as described previously [10,11]. Briefly, tumor cells were maintained in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. To generate spheroids, cells were plated in agar-coated culture flask. After 3 days of culture, tumor spheroids with a diameter of approximately 250 μm had formed. For ALA/PDT, spheroid cultures were supplemented with 12.5 μg/ml ALA (Merck, Darmstadt, Germany) and incubated for 4 h. Spheroids were collected under microscopic control and transferred (25 spheroids/well) into agar-coated flat-bottom 96-well plates (Greiner) containing 100 μl/well of DMEM without phenol red. Exposure to laser light was performed for 625 s with an energy of 30 mW/cm^2 (equivalent to 25 J/s or 1 W on 33 cm^2) using a Ceralas 633-nm PDT diode laser (Biolitec, Jena, Germany). After laser light exposure, spheroids were used in the experiments. These ALA/PDT conditions result in induction of apoptosis in about 60% of cells [11]. Untreated spheroids and spheroids treated with ALA only or exposed to laser-light only served as controls.

2.3. Under-agarose chemotaxis assay

Chemoattraction of MSC by glioblastoma spheroids was studied using an under-agarose chemotaxis assay as described [14,15]. Briefly, 0.8% agarose (Eurogentec, Cologne, Germany) in PBS was boiled, mixed after cooling with 0.5% bovine serum albumin (BSA; Roth, Karlsruhe, Germany) in DMEM and poured into 6-well plates (Costar/Corning, Wiesbaden, Germany). Three 2 mm wide and 5 mm long slots 5 mm apart from each other were cut in the agarose of each well. 8×10^4 MSC were then added to the central slots of each well, 70 μl of chemoattractant (25 treated or untreated spheroids) in the left target slots and 70 μl of 0.5% BSA in DMEM in the right control slots. The number of cells, which migrated towards the target slot subtracted by the number of cells, which migrated towards the control slot, was determined for each well after a 12 h migration period.

To study the contribution of HGF to MSC migration towards the spheroids, neutralization studies were performed by adding neutralizing anti-HGF polyclonal antibody (2 μg/ml; goat IgG; R&D Systems, Wiesbaden) or normal goat IgG (2 μg/ml; Santa Cruz, Heidelberg, Germany) to the targets.

2.4. Detection of MET and HGF expression

MET expression on MSC was determined by flow cytometry using an anti-MET monoclonal antibody (5 μg/ml; clone 95106, IgG1; R&D Systems) and Fluorescein isothiocyanate (FITC)-conjugated F(ab)2-goat-anti-mouse IgG + M (Beckman-Coulter, Krefeld, Germany) as secondary antibody. Cells were analyzed on a FACS Canto flow cytometer (BD Biosciences, Heidelberg, Germany).

Intracellular HGF and apoptosis were detected by double labeling with FITC-conjugated Annexin V (Beckman-Coulter) and anti-HGF rabbit polyclonal antibody (15 μg/ml; Abgent, San Diego, CA) 12 h after ALA/PDT or control treatments. Phycoerythrin (PE)-conjugated goat-anti-rabbit IgG (Jackson Immuno Research, Newmarket, UK) served as secondary antibody for anti-HGF. Cells were stained first with Annexin V according to the manufacturer's protocol. Subsequently, they were fixed and permeabilized using the IntraPrep Fix/Perm kit (Beckman-Coulter), stained with anti-HGF polyclonal antibody and finally labeled with the secondary antibody prior to flow cytometric analysis.

HGF protein in conditioned media of glioblastoma spheroids obtained 12 h after ALA/PDT or control treatments was quantified by enzyme-linked immunosorbent assay (ELISA; Shino Test Corporation, Kanagawa, Japan) according to the manufacturer's protocol.

Detection of HGF transcripts by reverse transcription-polymerase chain reaction (RT-PCR) was performed as described recently [14,15]. Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) served as positive control.

2.5. Statistical analysis

All data are presented as mean ± SEM for $n \geqslant 3$ unless stated otherwise. Statistical significance was determined with the Student's *t*-test using Prism Software (GraphPad, San Diego, CA).

3. Results

3.1. Attraction of MSC by glioblastoma spheroids is enhanced by ALA/PDT

To assess migration of MSC towards U87 and U251 glioblastoma spheroids, an under-agarose migration assay was performed. In three independent experiments using different MSC preparations, MSC migrated towards the spheroids (Fig. 1). Migration was significantly enhanced after ALA/PDT compared to the untreated spheroids (U87, 3.6 ± 0.6-fold, $p = 0.0096$; U251, 3.8 ± 0.4-fold, $p = 0.0018$). In contrast, control spheroids treated with exposure to laser light or incubation with ALA only showed no such effect.

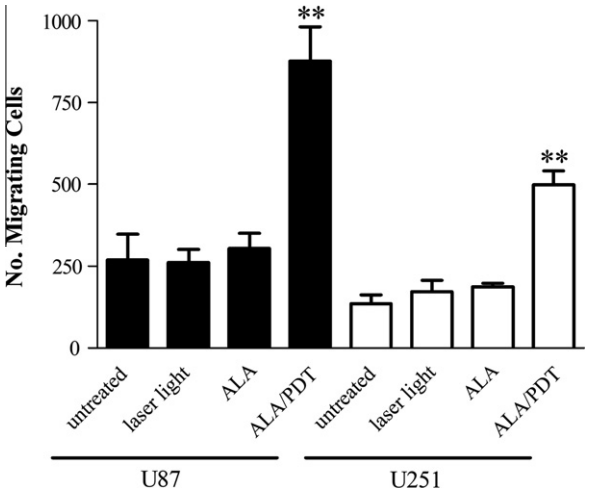

Fig. 1. ALA/PDT enhances migration of MSC towards glioblastoma spheroids. Migration of MSC towards untreated, laser light-exposed, ALA-treated or ALA/PDT-treated spheroids of the glioblastoma cell lines U87 and U251 was assessed in an under-agarose migration assay. The total number of specifically migrating MSC was determined after a 12-h migration period. Data are presented as mean ± SEM of three independent experiments. Statistical significance for the comparison with untreated spheroids is indicated, $^{**}p \leqslant 0.001$.

3.2. ALA/PDT up-regulates HGF production in glioblastoma spheroids

MSC expressed MET, the receptor for HGF, as determined by flow cytometry (Fig. 2A). RT-PCR analysis of the glioblastoma cell lines revealed expression of HGF (Fig. 2B) consistent with the detection of significant HGF protein levels in medium conditioned by untreated spheroids (Fig. 2C; U87, 5.6 ± 0.03 ng/ml; U251, 2.9 ± 0.13 ng/ml). ALA/PDT resulted in a significant increase in HGF release by the spheroids (U87, 2.6 ± 0.1-fold, $p = 0.0028$; U251, 3.1 ± 0.1-fold, $p = 0.0016$) compared to the untreated spheroids, whereas treatment with exposure to laser light or incubation with ALA only had no or only minimal effects. The presence of HGF in untreated living cells was also confirmed by intracellular staining (Fig. 2D and F). After ALA/PDT of spheroids, Annexin V staining indicated induction of apoptosis (Fig. 2E and G). HGF was present in living as well as apoptotic cells with a tendency of higher levels overall (Fig. 2E) or in apoptotic compared to living cells (Fig. 2G) after ALA/PDT.

3.3. Attraction of MSC by glioblastoma cells is mediated by HGF

To determine whether the HGF/MET axis plays a role in the tropism of MSC for glioblastoma cells and its enhancement by ALA/PDT, migration assays were performed in the presence of a neutralizing polyclonal anti-HGF antibody. Neutralizing HGF significantly inhibited migration of MSC towards U87 and U251 spheroids, irrespective of whether they were untreated or treated with laser light, ALA or ALA/PDT (Fig. 3). Inhibition was nearly complete, reaching 80.2 ± 3.1% and 74.2 ± 3.1% inhibition for ALA/PDT-treated U87 and U251 spheroids, respectively. In contrast, treatment with a control antibody had no inhibitory effects.

4. Discussion

Tropism of MSC for glioblastomas is well established. Irrespective of whether injected into the ipsilateral or contralateral carotid artery [26] or possibly even following systemic application [27], MSC have been shown to home to the tumor, to localize between the tumor and the normal brain parenchyma, and to infiltrate the

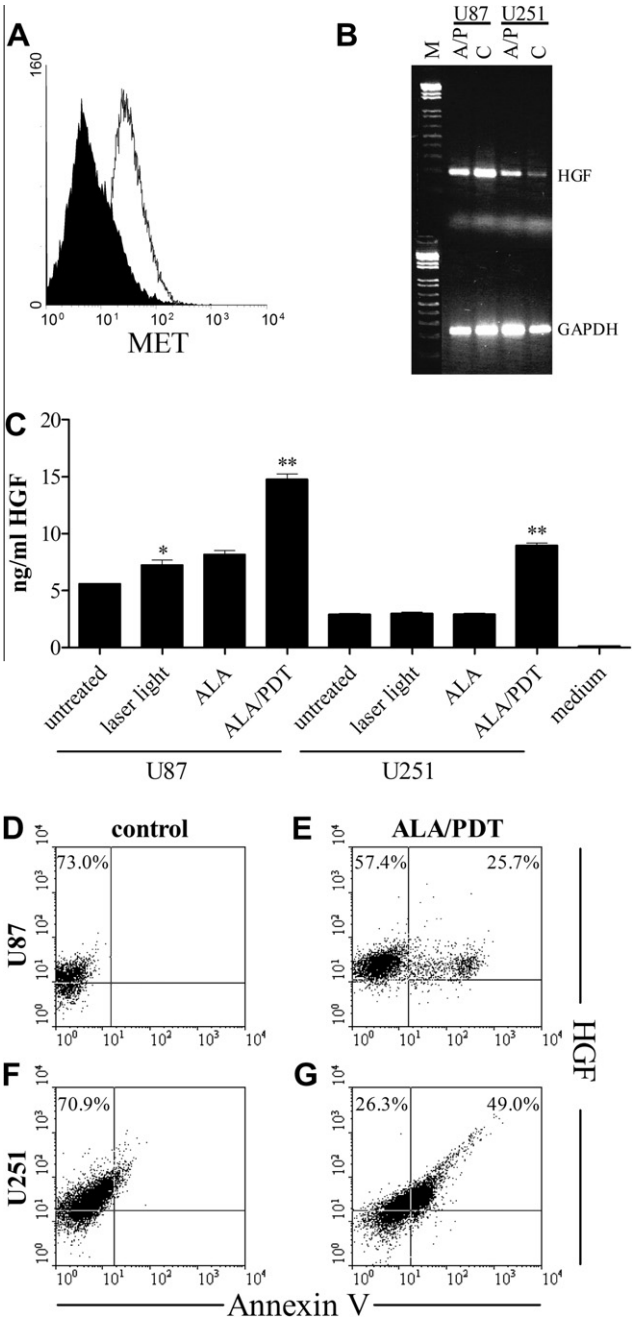

Fig. 2. ALA/PDT up-regulates HGF production in glioblastoma spheroids. Expression of the HGF receptor, MET, on MSC was determined by flow cytometry (A). Data of one of three independent experiments are shown. Cells were labeled with a MET-specific monoclonal antibody (open histogram) or an isotype control antibody (black histogram). The presence of HGF transcripts in untreated and ALA/PDT-treated U87 and U251 spheroids was determined by RT-PCR (B). GAPDH served as positive control, reactions without the addition of reverse transcriptase as negative controls (all negative, data not shown). M indicates the 100-bp ladder size marker. HGF protein levels in conditioned media derived from untreated, laser light-exposed, ALA-treated or ALA/PDT-treated spheroids of the glioblastoma cell lines U87 and U251 were determined by ELISA (C). Data represent mean ± SEM of two independent experiments. Statistical significance for the comparison with untreated spheroids is indicated, $^{**}p \leqslant 0.001$; $^{*}p \leqslant 0.05$. Apoptosis and the intracellular presence of HGF in living as well as apoptotic cells was detected by double labeling of untreated (D, F) and ALA/PDT-treated (E, G) U87 (D, E) and U251 spheroids (F, G) with Annexin V and anti-HGF antibody and flow cytometric analysis. Quadrants were set according to unlabeled controls.

tumor bed. Here, we have identified HGF as a major factor contributing to the migration of MSC towards glioblastoma cells. Moreover, we have shown that ALA/PDT of glioblastoma spheroids

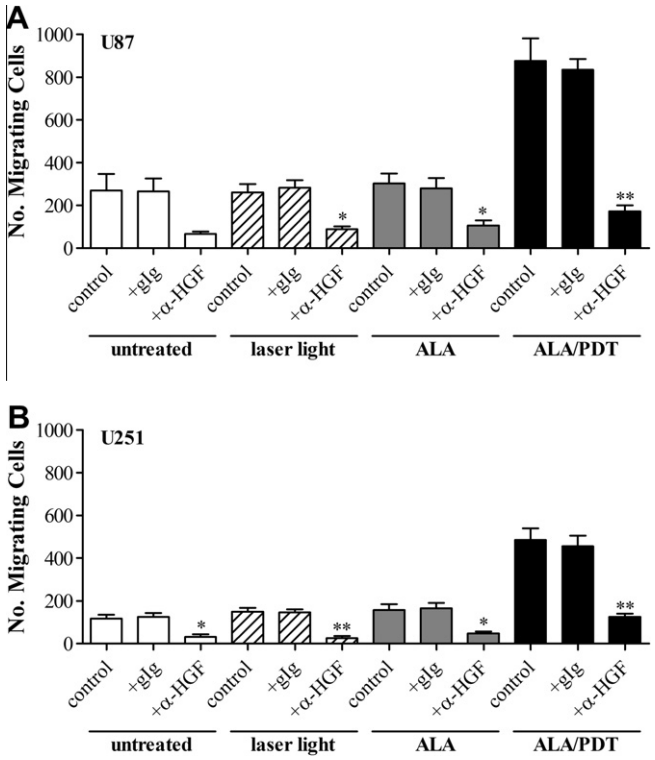

Fig. 3. MSC migrate towards glioblastoma spheroids in an HGF-dependent fashion. Migration of MSC towards untreated (open bars), laser light-exposed (hatched bars), ALA-treated (grey bars) or ALA/PDT-treated spheroids (black bars) of the glioblastoma cell lines U87 (A) and U251 (B) in the presence of a neutralizing polyclonal anti-HGF antibody or control IgG was determined. Data are presented as mean ± SEM of three independent experiments. Statistical significance for comparison with untreated and treated spheroids in the absence of antibodies is indicated, $^{**}p \leqslant 0.001$; $^{*}p \leqslant 0.05$.

induces apoptosis, augments HGF release and thereby causes an increase in MSC attraction.

Expression of HGF in glioblastoma cells, including U87 and U251 cell lines, has been described before [17,19,28,29]. Moreover, Chu et al. reported increased HGF production following gamma irradiation or hypoxia of U251 cells [29,30], and Kim and colleagues showed that irradiation of glioblastoma cells enhances attraction of MSC [31]. Thus, the ALA/PDT induced up-regulation of HGF in glioblastoma spheroids described here may represent a stress response, similar to that in response to gamma irradiation and hypoxia [29–31] and to the induction of apoptosis in neurons, which results in attraction of MSC in an HGF-dependent fashion [14]. Indeed, we could show recently [11] and confirm here that ALA/PDT of glioblastoma spheroids causes apoptosis, thus the type of cell death, which in neurons is associated with production of HGF. Tropism of other stem cell populations for glioblastomas appears to be regulated similarly: hematopoietic stem and progenitor cells [32] and neural stem cells [33] show enhanced migration towards glioblastoma cells *in vitro* as well as *in vivo* when these had been previously irradiated or subjected to hypoxic conditions before. Such stress responses, however, may also result in attraction of immune cells: we could recently show migration of immature dendritic cells towards ALA/PDT-treated glioma spheroids [10]. Thus, stress responses appear to initiate a concerted action by recruiting various cell types with regenerative or immunological activities. Interestingly, ALA/PDT appears to affect HGF release not only by the apoptotic, but also by the surviving cells. Whether this effect on surviving cells is direct or mediated indirectly by neighboring apoptotic cells requires further investigations.

Tropism of hematopoietic stem and progenitor cells for glioblastomas appears to be mediated by the CXCL12/CXCR4 axis [32], whereas for neural stem cells the CXCL12/CXCR4, vascular endothelial growth factor/vascular endothelial growth factor receptor 2 (VEGF/VEGFR2), urokinase-type plasminogen activator/urokinase-type plasminogen activator receptor and the HGF/MET axes appear to contribute [33]. HGF has been described as chemoattractant for MSC [14,22] as well as other cell types including microglia cells [34], neural stem cells [33,35] and glioblastoma cells [17,36]. However, its role in driving migration of MSC towards glioblastomas has not been addressed before, and tropism has been attributed to other factors produced by glioblastoma cells, particularly VEGF [37], platelet-derived growth factor [38,39], CXCL12 [40] and CXCL-8 [31,41]. MSC express a multitude of chemokine and growth factor receptors involved in chemoattraction [42], some of which are expressed on subsets of cells only like CXCR4, CCR1 and CX3CR1 [43]. Moreover, there may be an interplay between the individual pathways, e.g. Tu et al. reported an up-regulation of CXCL12 in U251 glioblastoma cells after stimulation with HGF [44]. Thus, several pathways may contribute to the tropism of MSC for glioblastomas. So far, our observations are limited to *in vitro* studies and the analysis of cell lines. Although a three-dimensional spheroid model has been used, which mimics micro tumors more closely than monolayer cultures [45], differences between cell lines and primary tumor cells as well as differences in the micro milieu of the tumor *in vivo* may influence the results. Therefore, the effect of ALA/PDT on the HGF-driven tropism of MSC for glioblastomas has to be confirmed *in vivo*.

The increasing local concentration of HGF following ALA/PDT in glioblastomas and attraction of MSC to the tumors may have beneficial as well as detrimental effects. HGF contributes to glioblastoma growth, migration and invasion, and has cytoprotective, anti-apoptotic, immunosuppressive and angiogenic activity [16–19,46]. However, we could document that irrespective of the HGF expression described here, ALA/PDT causes a long-lasting inhibition of glioblastoma cell migration and invasiveness [11] and promotes anti-tumor immunity by initiating the initial steps of an adaptive immune response [10]. Moreover, Xie et al. have recently shown that expression of HGF by glioblastoma cells correlates with high levels of MET phosphorylation and predicts their sensitivity to therapeutic inhibition of the MET pathway [47]. Thus, there may be a rational for combining ALA/PDT with MET–HGF targeted therapy. Similarly, the recruitment of MSC by HGF may also have positive and negative consequences. They may contribute to angiogenesis, immunosuppression and tumor cell survival [48], but may also induce glioblastoma cell death directly [39]. Moreover, MSC are used to deliver therapeutics such as cytokines, enzymes/pro-drugs, oncolytic viruses and toxins to glioblastomas [25], and this strategy may be improved when combined with ALA/PDT, due to an enhanced tropism of the MSC. Although the net effect of ALA/PDT is currently unknown and requires further analyses, the beneficial effects of ALA/PDT may prevail as suggested by early clinical studies [3,4].

In summary, we have shown that HGF contributes to the attraction of MSC towards glioblastoma cells and that ALA/PDT enhances HGF production resulting in increased migration of MSC, which may have direct therapeutic consequences or may be exploited to improve targeted gene therapy with MSC serving as therapeutic vectors.

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4 Diskussion

Es konnte gezeigt werden, dass die HGF/MET-Achse eine zentrale Bedeutung für die Rekrutierung von MSC zu geschädigten Kardiomyozyten und neuronalen Zellen hat. Auch für die Migration von MSC zu Glioblastomzellen spielt sie eine entscheidende Rolle. Apoptose, in Herz- und Nervenzellen direkt induziert, in GBM-Zellen durch ALA/PDT verursacht, konnte in direkten Zusammenhang mit diesen HGF-vermittelten Tropismen gebracht werden, was aufgrund der geweberegenerativen und gentherapeutisch ausnutzbaren Eigenschaften von MSC klinische Relevanz haben kann.

Die Rekrutierung von MSC zu geschädigtem Gewebe [37] mit anschließender Ausübung regenerierender Aktivität, die wahrscheinlich eher auf parakrinen Mechanismen und nicht auf einer gewebespezifischen Differenzierung beruht [10-11], ist in der Literatur ausführlich beschrieben. Auch die Bildung und proteolytische Aktivierung von HGF im Zusammenhang mit Gewebeschäden ist bekannt [50, 53]. HGF wird bei ischämischer Herz- [54] und Hirnschädigung [55] vermehrt gebildet und hat eine kardio- [56] und neuroprotektive [57] Wirkung aufgrund von anti-apoptotischen [58-59] und pro-angiogenetischen [60, 77] Eigenschaften. Weiterhin wurde die Rekrutierung von MSC [49-51], unrestringierten somatischen Stammzellen [67], endothelialen Progenitorzellen [78], kardialen [79] sowie neuralen Stammzellen [80] in der Literatur mit der HGF/MET-Achse in Verbindung gebracht. Somit trägt die Migration solcher Vorläuferzellen zu Gewebeschäden zusätzlich zur direkten zytoprotektiven Wirkung von HGF vermutlich zur Regeneration bei. Eine Steigerung der Effektivität des HGF-vermittelten Tropismus von MSC, welche ein erstrebenswertes Ziel bei deren Transplantation im Hinblick auf regenerative Therapien darstellt, ließe sich möglicherweise durch verstärkte Produktion von HGF im geschädigten Milieu erzielen oder durch erhöhte Expression von MET auf MSC. Tatsächlich konnten Rosova et al. zeigen, dass hypoxische Bedingungen bei der Kultivierung von MSC zu einer verstärkten Expression von MET führten, und eine kritische Ischämie *in vivo* assoziert war mit lokal erhöhtem HGF. Beides zusammen trug zu einer verbesserten Migration der MSC und einer beschleunigten Wiederherstellung der Durchblutungssituation bei [81].

In „*Hepatocyte growth factor-mediated attraction of mesenchymal stem cells for apoptotic neuronal and cardiomyocytic cells*“ produzierten ausschließlich apoptotische Zellen HGF und bewirkten eine HGF/MET-vermittelte Rekrutierung von MSC. Dies traf auf nekrotische und vitale Zellen nicht zu. Die Art des Zelltodes kann über unterschiedliche Signalwege somit zu einer fein regulierten Auslösung von Zelltropismen führen, die grundlegend verschiedene Konsequenzen für das Schicksal des geschädigten Gewebes haben können. HGF ist neben seinen erwähnten anti-apoptotischen Eigenschaften auch immunsuppressiv, was unter anderem durch Hemmung der Antigen-präsentierenden Aktivität von DC [82] und Induktion immunologischer Toleranz nach Herztransplantation im Mausmodell [83] gezeigt werden konnte. MSC besitzen neben ihrer geweberegenerierenden Aktivität [10] ebenfalls immunsupprimierende Eigenschaften [14]. Sie hemmen unter anderem die Reifung von DC [15] und deren zelluläre Sekretion von TNF-alpha [16]. Da TNF-alpha neben der Leukozyten-Rekrutierung auch an der Induktion von Apoptose durch Aktivierung von Todesrezeptoren beteiligt ist [84], steht dies im Einklang mit einer zusätzlichen anti-apoptotischen Aktivität von MSC [74]. Die Apoptose-induzierte Rekrutierung von MSC vermittelt durch HGF ist also in ein Setting einzuordnen, in dem Limitierung von Apoptose, Geweberegeneration und immunologische Toleranz vorherrscht.

In einem Mikromilieu, in dem vorwiegend nekrotischer Zelltod stattfindet, besteht eine grundlegend andere Situation. Nekrose ist assoziiert mit einer Vielzahl von immunstimulierenden Signalen, wie beispielsweise *high mobility group box* 1 (HMGB1), ein Kernprotein, das entweder passiv von nekrotischen Zellen freigesetzt [85] oder aktiv unter anderem von Monozyten und Makrophagen nach vorheriger Stimulation sezerniert wird [86-87]. HMGB1 und andere immunstimulierende Mediatoren aus sterbenden Zellen, sogenannte *danger-associated molecular patterns*, kurz DAMP [88], führen zu einer Rekrutierung von Immunzellen, wie beispielsweise DC [89]. Da in der ersten Studie der vorliegenden Arbeit die Induktion von nekrotischem Zelltod zu keiner Migration von MSC führte, einhergehend mit dem Ausbleiben einer Produktion von HGF, liegt die Annahme nahe, dass das entsprechende Mikromilieu bei Nekrose einen Weg Richtung Immunität einschlägt, in dem geweberegenerierende und den

zellulären Schaden begrenzende Antworten in den Hintergrund geraten. Tatsächlich konnte kürzlich bestätigt werden, dass nekrotischer Zelltod über die Freisetzung von HMGB1 Monozyten und DC rekrutiert (Manuskript in Vorbereitung). Interessanterweise beschreiben Meng et al. die Migration von MSC zu HMGB1 [90]. Da die Freisetzung von HMGB1 typischerweise mit nekrotischem Zelltod in Verbindung gebracht wird [85], suggeriert die Studie von Meng et al. somit einen möglichen Zusammenhang zwischen Nekrose und der Migrationsinduktion von MSC. Andererseits wurde in einer neueren Studie die Freisetzung von HMGB1 auch aus apoptotischen Zellen beschrieben. In diesem Fall war HMGB1 jedoch durch mitochondriale reaktive Sauerstoffspezies oxidiert und somit immunologisch inaktiviert worden [91]. Der Einfluss von HMGB1 und anderen DAMP auf einen möglichen Migrationsanstoß von MSC ist im jeweiligen Mikromilieu daher nur schwer vorhersehbar. Keine der drei in der vorliegenden Arbeit gewählten Bedingungen für die Induktion von Nekrose (H_2O_2, Hitze, Ethanol) resultierten in einer Migration von MSC. Kulturen von MSC können desweiteren verschiedene Subpopulation beinhalten, die auf unterschiedliche Migrationssignale unterschiedlich stark (oder eben gar nicht) reagieren können. Die im Rahmen dieser Arbeit verwendeten MSC-Kulturen waren durchweg positiv für MET. Da in den HGF-Inhibitionsstudien dieser Arbeit jedoch niemals eine vollständige Hemmung der MSC-Migration erreicht wurde, könnte dies ein Hinweis dafür sein, dass ein Teil der Zellen trotz der Anwesenheit migrationsinduzierender Mediatoren nicht rekrutiert wird. Für die Migration von MSC zu geschädigtem Herz [92] und Hirn [93] stellt die SDF-1/CXCR4-Interaktion einen alternativen Signalweg dar. Der Rezeptor CXCR4 lässt sich jedoch nur auf Subpopulationen von MSC nachweisen, was auch auf andere Chemokin-Rezeptoren, wie CCR1 oder CX3CR1, zutrifft [94]. Das Zusammenspiel von chemotaktischen Signalen für MSC erscheint somit komplex und ist unzureichend verstanden.

In „*Migration of mesenchymal stem cells towards glioblastoma cells depends on hepatocyte-growth factor and is enhanced by aminolaevulinic acid-mediated photodynamic treatment*" wurde gezeigt, dass MSC HGF-abhängig zu Glioblastomzellen migrieren. Dies konnte durch ALA/PDT-Behandlung der

GBM-Zellen signifikant verstärkt werden, was mit einer Induktion von Apoptose und einer vermehrten Expression von HGF durch die Tumorzellen einherging.

Die Migration von MSC zum GBM nach deren Injektion in die Arteria carotis [42], systemisch in eine Vene [95] oder intranasal appliziert [96] ist in der Literatur gut dokumentiert. Auch die Expression von HGF in GBM-Zellen ist bekannt [61-62] und lässt sich durch Bestrahlung oder hypoxische Bedingungen noch steigern [97-98]. In diesem Zusammenhang kann die ALA/PDT-verursachte erhöhte Freisetzung von HGF als Bestandteil einer Stress-Antwort interpretiert werden, analog zu der nach Bestrahlung und Hypoxie [97-98] oder nach Induktion von Apoptose in Nervenzellen [67]. Eine Konsequenz dieser Stressantwort stellt die Rekrutierung von MSC dar.

Aufgrund der immunsupprimierenden Wirkung von HGF [82-83] und MSC [14] würde man in diesem Setting eigentlich erwarten, dass immunologische Toleranz nach ALA/PDT im GBM vorherrscht. Es kommt jedoch zu einer akuten Entzündungsreaktion mit Hitzeschockprotein-abhängiger Einwanderung von Leukozyten in den Tumor und einer Aktivierung von DC, welche in einer antitumoralen Immunantwort resultiert [99-100]. Ob und inwiefern die pro-inflammatorische Antwort nach ALA/PDT in Konkurrenz steht zu potentiell immunhemmenden Effekten durch die Einwanderung von MSC in den Tumor, muss daher in weiteren Studien geklärt werden. Interessanterweise gibt es auch einen immunogenen apoptotischen Zelltod, wie es beispielsweise bei der Induktion von Apoptose von Tumorzellen mittels Antrazyklinen gezeigt wurde [101]. Auch ALA/PDT verursacht Apoptose im GBM [70], und die *in vitro*-Untersuchungen von Etminan et al. weisen auf eine immunogene Form der ALA/PDT-induzierten Apoptose hin [100]. Desweiteren werden in der Literatur neben den bereits beschriebenen immuninhibierenden Effekten von HGF kontroverserweise auch immunstimulierende Aktivitäten dokumentiert. Insbesondere eine fördernde Wirkung von HGF auf die Rekrutierung von Monozyten [102] und T-Zellen [103] wurde berichtet. Die beobachtete gesteigerte HGF-Produktion in der zweiten Studie der vorliegenden Arbeit könnte so also auch im Zusammenhang stehen mit der beschriebenen Immunantwort auf den Tumor nach ALA/PDT.

HGF fördert, insbesondere durch seine pro-angiogenetischen [60, 77] und auf GBM-Zellen zytoprotektiven Effekte [64], Wachstum und Invasion des GBM [61, 63]. Weiterhin induziert es die Expression von Matrix-Metalloproteinase (MMP)-2 in GBM-Zellen [104], was ebenfalls der Invasion des Tumors zugutekommen kann. Da in der vorliegenden Arbeit eine ALA/PDT-Behandlung von GBM-Zellen mit einer erhöhten HGF-Freisetzung einherging, suggerieren diese Daten potentiell tumorfördernde Effekte nach photodynamischer Behandlung. Ein direkter therapeutischer Nutzen von ALA/PDT bei Patienten mit GBM ist jedoch wahrscheinlich [105-106]. Außerdem wurden tumorhemmende Effekte von ALA/PDT in Verbindung gebracht mit einer verminderten Expression von MMP-7 und MMP-8 [69]. Gründe für die scheinbar im Konflikt stehenden Daten könnten darin bestehen, dass die beobachtete Hemmung von MMP-7 und MMP-8 nach ALA/PDT in einem Setting unabhängig von HGF stattfindet. Dies würde bedeuten, dass der fördernde Einfluss von HGF auf MMP nach ALA/PDT-Behandlung eine untergeordnete Rolle spielt, beispielsweise durch andere ALA/PDT-induzierte Mechanismen, die mit HGF und MMP interferieren. Zum anderen ist es möglich, dass HGF und ALA/PDT auf jeweils verschiedene Subtypen von MMP in den Tumorzellen wirken; Interferenzen in der Wirkung dieser Subtypen untereinander sind nicht auszuschließen. Obwohl nachteilige Effekte durch eine gesteigerte Produktion von HGF in den GBM-Zellen nach ALA/PDT möglich, und weitere Studien deshalb notwendig sind, kann eine anti-tumorale Wirkung der HGF-vermittelten verstärkten Migration von MSC zum Tumor überwiegen, sei sie durch gentherapeutisch modifizierte MSC, die beispielsweise IFN-beta oder TRAIL exprimieren [35], oder durch eine direkte zytotoxische Wirkung auf den Tumor [43] hervorgerufen.

Apoptotischer Zelltod und der HGF/MET Signalweg spielen also eine Schlüsselrolle in der Rekrutierung von MSC. Dies kann von Relevanz sein für die Verwendung von MSC in der regenerativen Medizin wie auch in der zellulären Therapie von Glioblastomen.

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Eidesstattliche Versicherung

Ich versichere an Eides statt, dass die Dissertation selbständig und ohne unzulässige fremde Hilfe erstellt worden ist, und die hier vorgelegte Dissertation nicht von einer anderen medizinischen Fakultät abgelehnt worden ist.

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