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Matrix metalloproteinase-2 is involved in the migration and network formation of enteric neural crest-derived cells

RICHARD B. ANDERSON*

Department of Anatomy & Cell Biology, University of Melbourne, Victoria, Australia

ABSTRACT The enteric nervous system is derived from neural crest cells that emigrate from the hindbrain, enter the foregut and colonise the entire length of the gastrointestinal tract. Previous studies have shown that although enteric neural crest-derived cells migrate in chains, they have the ability to detach from their existing chain in order to join or form a new chain. In this study, the possible role of matrix metalloproteinase-3, -8 and -2/-9 on the migration of enteric neural crest-derived cells and the formation of the neural network within the developing gut were examined using specific pharmacological inhibitors. Blocking MMP-2/MMP-9 activity significantly decreased the distance that enteric neural crest-derived cells migrated through the developing gut. Morevover, the reticulated network formed by these cells was less complex. MMP-3 and MMP-8 inhibitors had no effect on neural crest migration. Expression studies showed that MMP-2, but not MMP-9, was expressed within the developing mouse gut. Collectively, the data suggest that MMP-2 activity is important for enteric neural crest-derived cell migration and the formation of the neural crest network.

KEY WORDS: *neural crest, cell migration, development, enteric nervous system*

Introduction

The formation of an enteric nervous system (ENS) requires the coordinated proliferation, migration, differentiation and survival of enteric neural crest-derived cells within the developing gut. During development, vagal level neural crest cells emigrate from the caudal hindbrain, enter the foregut and then migrate from rostral-to-caudal along the entire length of the gastrointestinal tract (Yntema and Hammond, 1954). Sacral level neural crest cells also make a small contribution to the ENS in the distal gut (Burns, 2000, Kapur, 2000, Anderson *et al.*, 2006b). Within the developing gut, enteric neural crest-derived cells have been shown to migrate in chains (Conner *et al.*, 2003, Young *et al.*, 2004, Druckenbrod and Epstein, 2005, Anderson *et al.*, 2006c). As these migrating chains advance along the gut, they form a reticulated network that precedes the formation of ganglia characteristic on the ENS (Anderson *et al.*, 2006c, Druckenbrod and Epstein, 2007). The process of neural crest cells migrating in chains is not unique to enteric neural crest-derived cells, as cranial, cardiac and trunk neural crest-derived cells have all been shown to migrate in chains during development (Kulesa and Fraser, 1998, Poelmann *et al.*, 1998, Kulesa and Fraser, 2000, Teddy and Kulesa, 2004, Kasemeier-Kulesa *et al.*, 2005).

The existence of chains of neural crest-derived cells within the developing gut suggests the presence of adhesive interactions. A variety of cell adhesion molecules, such as L1, have been reported to be expressed by the developing ENS (Chalazonitis *et al.*, 1997, Ikawa *et al.*, 1997, Yoneda *et al.*, 2001, Anderson *et al.*, 2006c), and it has been proposed that these molecules are responsible for the formation of chains of neural crest-derived cells (Young *et al.*, 2004, Anderson *et al.*, 2006c). Inhibition of L1 function increases the frequency at which individual neural crest-derived cells detach from chains and form solitary cells, and there is a concomitant reduction in the speed of migration (Anderson *et al.*, 2006c).

Time-lapse imaging studies have shown that in addition to maintaining strong adhesive interacts with each other, enteric neural crest-derived cells must also be able to move past each

Abbreviations used in this paper: GFP, green fluorescent protein; ENS, enteric nervous system; MMP, matrix metalloproteinases; N-bltm, N-t-butoxycarbonyl-L-leucyl-L-tryptophan methylamide; MTH, 2-4-Methoxybenzenesulfonyl-1,2,3,4-tetrahydroisoquinoline-3-hydroxamate; DMSO, dimethyl sulphoxide.

***Address correspondence to:** Richard B. Anderson. Department of Anatomy & Cell Biology, University of Melbourne, 3010, VIC, Australia. Fax: 61-3-9347-5219. e-mail: r.anderson@unimelb.edu.au

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other in order to migrate and to detach from each other during the formation of a new chain (Young *et al.*, 2004, Anderson *et al.*, 2006c). One group of molecules that have been shown to cleave cell adhesion molecules are the matrix metalloproteinases (MMPs) (Beer *et al.*, 1999, Galko and Tessier-Lavigne, 2000, Kajita *et al.*, 2001, Mechtersheimer *et al.*, 2001, Noe *et al.*, 2001). MMPs are a subfamily of zinc-dependent proteinases, which belong to the metzincin superfamily. MMPs are involved in degrading and remodeling the extracellular matrix (Yong *et al.*, 2001), and have been implicated in many different aspects of development and disease. During development, MMPs have been shown to influence the migration of some populations of neural crest cells (Robbins *et al.*, 1999, Cai and Brauer, 2002). For example, cardiac neural crest-derived cells encounter MMPs in the extracellular matrix as they migrate towards the heart (Cai *et al.*, 2000), and blocking their activity significantly retards the migration of these cells *in vitro* and *in vivo* (Cai and Brauer, 2002).

Enteric neural crest-derived cells also require MMP activity for migration (Anderson *et al.*, 2006c). Pharmacological inhibition of endogenous MMPs, using the broad-spectrum hydroxamate-based MMP inhibitor, GM6001, significantly reduced the distance that enteric neural crest-derived cells migrated and the network formed by the cells was less complex (Anderson *et al.*, 2006c). As the hydroxamate-based inhibitor GM6001 is known to block the activity of MMP-3 (stromelysin 1), MMP-8 (collagenase 2), MMP-2 (gelatinase A) and MMP-9 (gelatinase B), it is not yet known which MMP is important for enteric neural crest-derived cell migration. In the current study, the effects of pharmacological inhibition of MMP-3, -8 and -2/-9 were examined to identify specific MMPs involved in enteric neural crest-derived cell migration. Inhibition of gelatinase activity (MMP-2 and MMP-9) significantly disrupted the migration of enteric neural crest-derived cells in explants of embryonic mouse gut, as well as the reticulated network formed by the cells. In contrast, inhibition of MMP-3 and MMP-8 had no detectable effect. MMP expression studies showed that MMP-2, but not MMP-9, was expressed within the developing mouse gut. Together, the data suggest that MMP-2 activity is important for enteric neural crest-derived cell migration and the formation of the neural crest network within the developing gut.

Results

MMP-2/MMP-9 is required for enteric neural crest-derived cell migration

To examine the effect of MMP inhibition on enteric neural crest-derived cell migration in intact segments of gut, explants of mid-plus hindgut from E11.5 $Ret^{TGM/+}$ mice were cultured in the presence of MMP-3, MMP-8 and MMP-2/-9 specific inhibitors for 24 hrs, and the distance from the ileo-caecal junction to the most distal GFP^+ cell measured. At the beginning of the culture period, the most caudal GFP^+ cell was located in the caecum or most proximal colon. When explants of gut were grown in the presence of control medium, medium containing DMSO vehicle alone, or N-t-butoxycarbonyl-L-leucyl-L-tryptophan methylamide (a compound related to GM6001 but lacks any proteinase inhibitor activity) GFP^+ neural crest cells were located in the distal hindgut (Fig. 1A, B, G). In contrast, when explants were grown in the presence of the broad-spectrum hydroxamate-based MMP inhibitor, GM6001, the most caudal GFP^+ neural crest cells were usually located in the middle of the hindgut (Fig. 1C). The most caudal GFP^+ cells were significantly closer to the ileocaecal junction in GM6001-treated explants than in control cultures (one-way ANOVA, $P < 0.001$; Fig. 1G). These data confirm our previous observations that endogenous MMP activity is required for the migration of enteric neural crest-derived cells during development (Anderson *et al.*, 2006c).

GM6001 is known to block the activity of MMP-3, MMP-8, MMP-2 and MMP-9 (Galardy *et al.*, 1994, Solorzano *et al.*, 1997). The effect of specific MMP-3, MMP-8 and MMP-2/MMP-9 inhibitors on enteric neural crest-derived cell migration was therefore examined. When explants of E11.5 gut were grown in the presence of 5 μM or 10 μM of the MMP-3 specific inhibitor, NNGH, neural crest migration was not affected; the caudal most GFP^+ neural crest-derived cell was located in the distal hindgut (Fig 1D), and the distance migrated from the ileocaecal junction was not significantly different from control cultures (one-way ANOVA; Fig. 1G). Similarly, after explants were cultured in the presence of 5 μM or 10 μM of a MMP-8 specific inhibitor, MTH, no effect on migration was detected (Fig 1E, G). Higher concentrations (50-100 μM) of NNGH and MTH were also found to have no effect on migration (data not shown). However, the distance that enteric neural crest-derived cells migrated was reduced in the presence of the MMP-2/MMP-9 specific inhibitor, SB-3CT. After explants of gut were cultured in the presence of 100 μM of SB-3CT, the most caudal GFP^+ neural crest cells were usually located in the middle of the hindgut (Fig. 1F) and were significantly closer to the ileocaecal junction than in control cultures (one-way ANOVA, $P < 0.001$; Fig. 1G). Neural crest cell migration was unaffected in the presence of 50 μM of the SB-3CT (Fig. 1G). To determine whether a difference in gut size between DMSO control and 100 μM SB-3CT-treated explants may contribute to the difference in the location of enteric neural crest-derived cells in the E11.5 gut, the diameter of the midgut, caecum and hindgut was measured. No significant differences in the diameters of the midgut, caecum or hindgut were found between DMSO control and 100 μM SB-3CT treated gut (unpaired *t* test; data not shown). Together, these results show that enteric neural crest-derived cell migration requires MMP-2/MMP-9 activity.

In order to determine whether the effects of SB-3CT were reversible, explants of E11.5 gut were grown in the presence of 100 μM SB-3CT for 24 hrs and then cultured for an additional 24 hrs in control medium. After 24 hrs in the presence of SB-3CT, GFP^+ neural crest cells were significantly closer to the ileocaecal junction than in control cultures. However, after 48 hrs in culture, no significant difference was detected in the distance migrated by enteric neural crest-derived cells in control and SB-3CT treated explants (unpaired *t* test; Fig. 1J), thus suggesting that the effects mediated by SB-3CT are reversible.

To determine whether the effect of SB-3CT on enteric neural crest-derived cell migration was due to changes in the rate of proliferation or cell death, immunohistochemistry was performed on E11.5 $Ret^{TGM/+}$ embryonic gut grown in catenary culture using antibodies against phospho-histone 3, which detects cells from late G2 to telophase, and activated caspase 3, a marker of apoptotic cells. No significant difference was detected in the percentage of GFP^+ cells that were phospho-histone 3^+ or activated caspase 3^+ between explants cultured in the presence of DMSO control and those cultured in the presence of 100 μM SB-

3CT (unpaired *t* test; Fig. 1H, I). Therefore, the reduced distance that enteric neural crest cells migrated in the presence of SB-3CT was not due to altered rates of neural crest cell proliferation or cell death.

In order to confirm the data obtained using the inhibitor SB-3CT, explants of gut from E11.5 $Ret^{TGM/+}$ mice were cultured in the presence of another MMP-2 and MM-9 inhibitor, CTT (Hehr *et al.*, 2005). E11.5 $Ret^{TGM/+}$ catenary cultures were grown for 24 hours in the presence of control medium or medium containing 400 μM CTT. In explants cultured in the presence of CTT, the most caudal GFP^+ neural crest-derived cells were significantly closer to the ileocecal junction than in control cultures (unpaired *t* test; Fig. 1K).

MMP-2/MMP-9 is required for neural crest network formation

In addition to affecting the rate of neural crest-derived cell migration, the broad-spectrum MMP inhibitor GM6001 changes the appearance of the neural crest network within the hindgut (Anderson *et al.*, 2006c). This network is established by chains of neural crest-derived cells as they traverse through the gut mesenchyme. When explants of gut were grown in the presence of control medium (Fig. 1A) or medium containing DMSO (Fig. 1B), neural crest-derived cells within the hindgut formed an extensive network composed of multiple thin (1 cells thick) chains of cells. However in the presence of GM6001, these chains appeared thicker and reduced in number (Fig. 1C). A similar disruption to the neural crest network was detected in the presence of MMP-2/MMP-9 inhibitors (Fig. 1F). Rather than forming an extensive lattice-like arrangement within the hindgut, neural crest-derived cells were often present within a few corpulent chains. Blocking the activity of either MMP-3 or MMP-8 using NNGH and MTH respectively, had no effect on the neural crest network formation within the hindgut (Fig. 1D, E).

To quantify the disruption to the neural crest network, the percentage of area occupied by the neural crest-derived cells and the diameter of the neural crest chains within a 100 μm^2 region at a distance of 200 μm from the most caudal neural crest-derived cell was determined. No significant difference in the percentage of area occupied by neural crest-derived cells was detected in explants grown in the presence of control medium, medium containing DMSO, MMP-3 or MMP-8 inhibitor (Fig 2A). However, in the presence of either GM6001, SB-3CT or CTT neural crest-

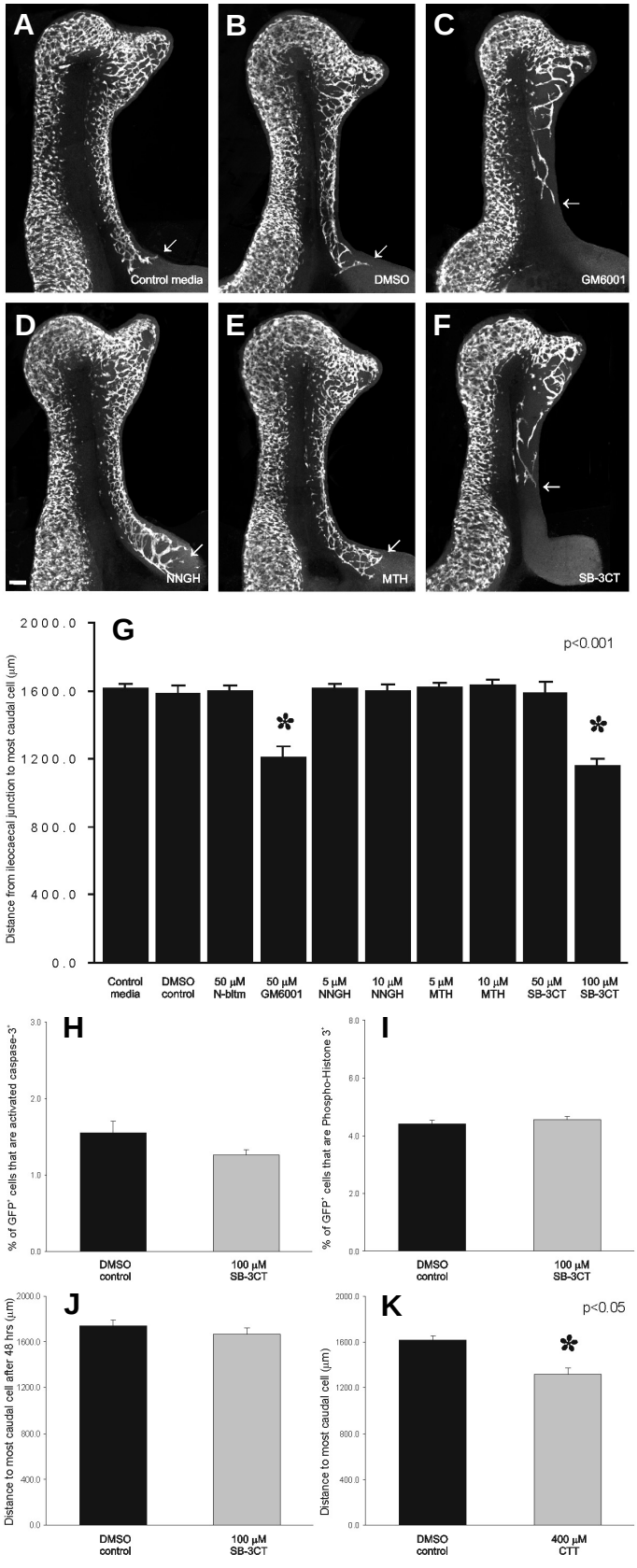

Fig. 1. Effect of blocking MMP activity on neural crest-derived cell migration. (A-F) *E11.5 $Ret^{TGM/+}$ gut cultured in the presence of control medium* **(A)**, *100 μM DMSO* **(B)**, *50 μM GM6001* **(C)**, *10 μM NNGH* **(D)**, *10 μM MTH* **(E)** *and 100 μM SB-3CT* **(F)**. *The most caudal neural crest-derived cells were significantly closer to the ileocaecal junction when cultured in the presence of GM6001 or SB-3CT. Arrows represent the location of the most caudal neural crest-derived cell.* **(G)** *Quantification of neural crest-derived cell migration when MMP activity was inhibited. The distance migrated by neural crest-derived cells was reduced when cultured in the presence of GM6001 or SB-3CT compared to control cultures.* **(H-I)** *Percentage of neural crest-derived cells undergoing cell death* **(H)** *or cell proliferation* **(I)** *in E11.5 gut cultured in the presence of DMSO control or SB-3CT. No significant difference was detected.* **(J)** *Quantification of E11.5 gut grown in the presence of DMSO control or SB-3CT for 24 hrs and then cultured for an additional 24 hrs in control medium. No significant difference was detected in the distance migrated by the enteric neural crest-derived cells after 48 hrs.* **(K)** *Quantification of neural crest location when cultured in control media or media containing CTT. The most caudal neural crest-derived cells were significantly closer to the ileocaecal junction when cultured in the presence of CTT. Scale bar, 100 μm.*

derived cells were found to occupy significantly less area than in control cultures (one-way ANOVA, $P < 0.05$; Fig. 2A). In addition, the diameter of the neural crest chains was larger in explants grown in the presence of either GM6001, SB-3CT or CTT compared to cultures grown in the presence of control medium, medium containing DMSO, MMP-3 or MMP-8 inhibitor (one-way ANOVA, $P < 0.05$; Fig. 2B).

The reduction in the complexity of the neural crest network in the hindgut is also reflected in changes in the number of times that neural crest-derived chains intersected with each other. No significant difference in the number of times that neural crest chains contacted each other within a 100 μm^2 region was detected in explants grown in the presence of control medium, medium containing DMSO, MMP-3 or MMP-8 inhibitor (Fig 2C). However, in the presence of either GM6001, SB-3CT or CTT the frequency at which chains of neural crest-derived cells intersected with each other was reduced compared to control cultures (one-way ANOVA, $P < 0.05$; Fig. 2C). Interestingly, no significant disruption to the neural crest network was detected in the caudal midgut between control and treated explants (one-way ANOVA; data not shown). Together, these results show that the newly-formed network created by migrating neural crest-derived cells requires MMP-2/MMP-9 activity, but that MMP-2/MMP-9 is not essential for the maintenance of the network behind the migration wavefront.

MMP-2 is expressed in the developing gut

The lack of specific inhibitors that selectively block MMP-2 or MMP-9 alone makes it difficult to determine whether one or both of these MMPs are involved in the migration of enteric neural crest-derived cells. Therefore, to examine whether MMP-2 and MMP-9 are expressed within the developing gut, gelatinase activity was assessed in solubilised samples of E11.5 gut. A 62 kD band was detected by gelatin zymogram, corresponding to MMP-2 (Fig. 2D). MMP-9 activity was not detected (n=3). EDTA is known to block all gelatinase activity. Therefore, to confirm that the 62 kD band is due to MMP activity, EDTA was added to the incubation buffer. The presence of EDTA completely abolished the band (Fig. 2E). These data show that MMP-2, but not MMP-9, is expressed within the developing mouse gut.

Discussion

Enteric neural crest-derived cell migration is known to be influenced by multiple factors, such as soluble molecules secreted by the gut mesenchyme (Anderson *et al.*, 2006a, Burns and Thapar, 2006, Heanue and Pachnis, 2007), cell surface molecules (Anderson *et al.*, 2006c) and extracellular matrix molecules (Breau *et al.*, 2006). In the current study, MMP-2 was also shown to be involved in enteric neural crest-derived cell migration and in the formation of the reticulated network within the developing mouse gut.

MMPs are a family of structurally related and highly conserved zinc-dependent endopeptidases, which are widely expressed within the developing nervous system (Yong *et al.*, 2001). MMPs were initially identified as potent regulators of the extracellular matrix (Yong *et al.*, 2001). They act to degrade the extracellular matrix, allowing cells to migrate through the dense extracellular meshwork. More recently, MMPs have also been shown to cleave cell surface molecules (Beer *et al.*, 1999, Galko and Tessier-Lavigne, 2000, Kajita *et al.*, 2001, Mechtersheimer *et al.*, 2001, Noe *et al.*, 2001). MMPs can disrupt cell-cell interactions by using a sheddase activity to cleave membrane bound molecules (Sternlicht and Werb, 2001).

There is now increasing evidence illustrating a role for MMPs in cell migration. Cardiac neural crest-derived cells have been shown to encounter extracellular matrix containing MMP-2 en route to the heart (Cai *et al.*, 2000) and blocking the activity of MMP-2 significantly decreased the distance migrated by these cells (Cai and Brauer, 2002). The migration of oligodendrocyte progenitors within the central nervous system is also impaired by treatment with MMP inhibitors (Amberger *et al.*, 1997). The inhibition of MMP activity significantly

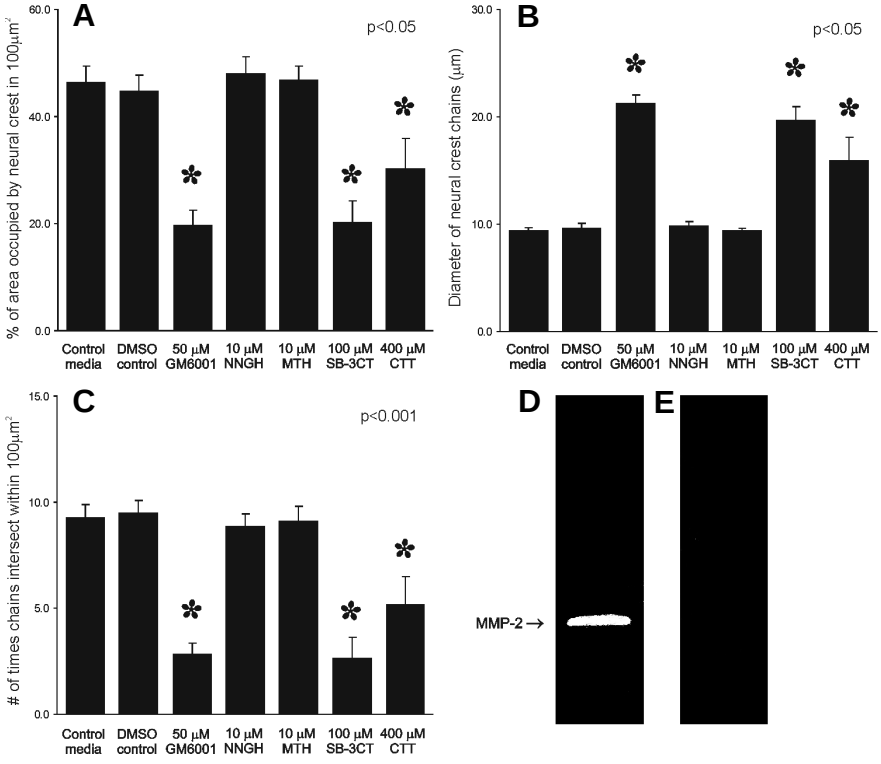

Fig. 2. Effect of blocking MMP activity on formation of the neural crest network. (A-C) *Quantification of the area occupied by neural crest-derived cells* **(A)**, *the diameter of neural crest chains* **(B)** *and the number of times they intersect* **(C)** *at a distance of 200 μm from the migratory wavefront. A significant difference in the area occupied by neural crest-derived cells, the diameter of the chains and the number of times they intersect with each other were detected when cultured in the presence of GM6001, SB-3CT or CTT.* **(D-E)** *Images of gelatinase zymograms from E11.5 gut. A band corresponding to MMP-2 was detected in tissue lysates derived from mouse gut* **(D)** *and is abolished in the presence of EDTA* **(E)**.

reduces the migratory ability of neuroblasts along the subventricular zone-olfactory bulb pathway (Bovetti *et al.*, 2007). In the current study, pharmacological inhibition of MMP-2/MMP-9, using either SB-3CT or CTT, significantly reduced the migratory ability of enteric neural crest-derived cells within the developing hindgut. It is likely that gelatinases are also involved in the migration of neural crest-derived cells within the midgut. However, due to technical reasons, it was not possible to examine this in the current study. Gelatine zymogram studies showed that MMP-2, but not MMP-9, is expressed within the E11.5 mouse gut, suggesting that MMP-2 is the likely metalloproteinase involved in enteric neural crest-derived cell migration at this age. SB-3CT has been shown to specifically inhibit MMP-2 and MMP-9 at 1000-fold lower concentrations than other MMPs (Hehr *et al.*, 2005), however it is possible that the concentrations of SB-3CT used in the current study may block other MMPs that are not classified as gelatinases. However, similar defects were also observed using another MMP-2 and MMP-9 inhibitor, CTT, suggesting that these effects are unlikely to be non-specific.

The mechanism by which MMP-2 mediates enteric neural crest-derived cell migration is not known. Disruptions to cell-cell adhesion have been reported to alter neural crest migration within the developing gut. Time-lapse observations showed that inhibiting the activity of the cell adhesion molecule, L1, significantly increased the frequency at which individual neural crest-derived cells broke away from their chains and formed solitary cells, resulting in delayed neural crest migration (Anderson *et al.*, 2006c). Therefore, it seems that enteric neural crest-derived cell migration requires a dynamic level of cell-cell adhesion. Migrating neural crest-derived cells must be able to attach, and then later detach in order to advance. One possibility is that MMP-2 may facilitate the detachment of neural crest-derived cells from existing chains. In the current study, neural crest-derived cells exposed to SB-3CT or CTT migrated as thick, multi-cell chains rather than thin, usually single cell chains observed in control preparations. The neural crest network present in SB-3CT or CTT treated preparations were also considerably less complex and occupied a significantly reduced area of the hindgut. This suggests that inhibiting MMP-2 activity may increase neural crest-derived cell-cell adhesion, preventing them from detaching from each other. However, we can not rule out the possibility that the thicker strands of neural crest-derived cells could be due to mechanisms unrelated to changes in cell adhesion.

Alternatively, SB-3CT could alter enteric neural crest-derived cell migration by reducing extracellular matrix degradation, and therefore reducing the invasiveness of the crest-derived cells. MMP inhibitors have been shown to block extracellular matrix degradation and migration of osteoblasts (Sato *et al.*, 1998) and impede the invasion of glioblastoma cells *in vitro* (Boghaert *et al.*, 1994). Enteric neural crest-derived cells have been shown to express various receptors for the extracellular matrix, such as α4, α6 and β1 integrins (Bixby *et al.*, 2002, Kruger *et al.*, 2002, Iwashita *et al.*, 2003, Breau *et al.*, 2006). Disruption to at least one of these integrins, β1 integrins, has been shown to alter enteric neural crest-derived cell migration (Breau *et al.*, 2006). The loss of β1 integrins on neural crest-derived cells resulted in the incomplete colonisation of the developing gut, as well as an abnormal organisation of the neural network (Breau *et al.*, 2006). Similar finding were observed in the current study, where blocking MMP-2/MMP-9 activity significantly reduced enteric neural crest-derived cells migration and altered the reticulated network formed by these cells. Interestingly, integrins have recently been reported to act as a receptor for MMPs (Bjorklund and Koivunen, 2005). This raises the possibility that β1 integrins and endogenous MMPs may interact within the developing gut. However, further studies are required to examine whether such interactions occur during enteric neural crest-derived cell migration.

In conclusion, this study shows that MMP-2 is important for the migration of neural crest-derived cells and the formation of the reticulated neural network within the developing hindgut. Further studies are required to elucidate the mechanisms by which MMP-2 mediates this effect.

Materials and Methods

Animals

In this study, Ret^{TGM} mice on a C57BL/6 background were used. The Ret^{TGM} mice have had cDNA encoding tau-EGFP-myc (TGM) inserted into the first coding exon of the *Ret* gene (Enomoto *et al.*, 2001) and all enteric neural crest-derived cells in the E11.5 gut express GFP (Young *et al.*, 2004). The genotype of adult Ret^{TGM} mice was determined by PCR using the primers and conditions as previously reported (Enomoto *et al.*, 2001). To distinguish $Ret^{TGM/+}$ from $Ret^{+/+}$ embryos, the stomach of each embryo was examined under a fluorescence microscope to determine if GFP^+ cells were present; only gut from $Ret^{TGM/+}$ embryos was used. Time-mated pregnant mice were anesthetised and killed by cervical dislocation and the embryos were removed. The morning on which a copulatory plug was observed was designated E0.5.

Catenary cultures

E11.5 $Ret^{TGM/+}$ mid- plus hindgut were cultured as previously described (Anderson *et al.*, 2006c). Briefly, guts were attached to the filter paper by the mesentery and suspended across a V-shaped notch which had been cut into 3x3 mm pieces of black Millipore paper (Millipore, Australia) sterilised in ethanol. Preparations were floated on 20 μl of tissue culture medium in a Terasaki well and cultured in a 5% CO_2 incubator at 37 °C for 24 or 48 hours. Explants were cultured in the presence of either unsupplemented culture medium (control medium) or in medium containing 5-400 μM dimethyl sulphoxide (DMSO; 0.1-0.4% v/v DMSO) vehicle; 50 μM broad-spectrum metalloproteinase inhibitor GM6001 (Hehr *et al.*, 2005, Anderson *et al.*, 2006c); Calbiochem, USA); 50 μM N-t-butoxycarbonyl-L-leucyl-L-tryptophan methylamide (N-bltm), a compound related to GM6001 but lacks any proteinase inhibitor activity (Galko and Tessier-Lavigne, 2000; Calbiochem); 5-10 μM of the MMP3 specific inhibitor NNGH (MacPherson *et al.*, 1997; Calbiochem); 5-10 μM of the MMP8 specific inhibitor 2-4-Methoxybenzenesulfonyl-1,2,3,4-tetrahydroisoquinoline-3-hydroxamate (MTH) (Matter *et al.*, 1999; Calbiochem); 50-100 μM of the MMP2/MMP9 specific inhibitor SB-3CT (Hehr *et al.*, 2005; Chemicon); 400 μM CTT (Hehr *et al.*, 2005; AnaSpec). Explants were cultured for 24 hours at then fixed in 4% paraformaldehyde in 0.1M phosphate buffer (PB) pH 7.2 and processed for immunohistochemistry.

Immunohistochemistry

Catenary cultures were processed for immunohistochemistry as previously described (Anderson *et al.*, 2007). Briefly, preparations were permeabilised in 1% Triton-X 100 for 30 minutes and then incubated in a goat anti-GFP polyclonal antibody (Rockland, USA) at 4 °C overnight, followed by a donkey anti-sheep FITC conjugated secondary antibody (Jackson Immunoresearch, USA) for 3 hours at room

temperature. Preparations were then mounted and viewed using either a BioRad 1024 confocal microscope or conventional fluorescence microscopy.

Zymogram protease assay

Gelatin-containing zymograms were performed as previously described (Cai *et al.*, 2000). Briefly, dissected E11.5 gut were homogenised in phosphate buffer and centrifuged at 10,000*g* for 10 minutes at 4 °C. The supernatant was then loaded onto a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel containing 0.1% gelatin. Equal quantities of protein were loaded into each lane. After electrophoresis, gels were washed with two changes of 2.5% Triton X-100 to remove the SDS and then incubated in reaction buffer (40 mM Tris-HCL, 10 mM $CaCl_2$, 0.02% thimerosal) for 24 hours at 37 °C. The gels were stained with Coomassie blue and destained with methanol/acetic acid/water (50:10:40). The electrophoretic mobility of proteins with enzymatic activity was revealed by the absence of stained gelatin. In some cases, 10 mM EDTA was added to the reaction buffer to block MMP activity. The gelatin-containing zymograms were then scanned on an Epson 4990 scanner.

Analysis of neural crest migration and morphology of the neural crest network

The distance migrated by the enteric neural crest-derived cells during the culture period was determined by measuring the distance between the ileocaecal junction and the most caudal neural crest-derived cell in the hindgut. The area occupied by the neural crest-derived cells, the number of times that neural crest chains intersected with each other and the diameter of the neural crest chains were all calculated using a 100 μm^2 box at a distance of 200 μm from the migratory wavefront. All measurements were made using ImageJ (NIH). A minimum of 8 preparations were used for each condition. The results are reported as mean ± standard error of the mean.

Analysis of cell proliferation and cell death

To determine the rate of neural crest-derived cell proliferation and cell death in the catenary cultures, immunohistochemistry was performed using antibodies against Phospho-Histone 3 (Upstate, USA) and activated caspase-3 (R&D Systems, USA) followed by a goat anti-rabbit Alexa 594 secondary antibody (Molecular Probes, USA). Preparations were viewed on a confocal microscope using a x63 lens. Images (single optical sections) of randomly chosen fields within the midgut and rostral hindgut were obtained, and the percentages of GFP^+ neural crest cells that were also Phospho-Histone 3^+ or activated caspase-3^+ were counted. Every GFP^+ cell in each field was examined. A minimum of 1000 GFP^+-neural crest cells were counted in total, with a minimum of 200 cells per preparation counted (n=4). Results are presented as mean ± SEM.

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