The type III transforming growth factor-β receptor inhibits proliferation, migration, and adhesion in human myeloma cells

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ABSTRACT Transforming growth factor-β (TGF-β) plays an important role in regulating hematopoiesis, inhibiting proliferation while stimulating differentiation when appropriate. We previously demonstrated that the type III TGF-β receptor (TβRIII, or betaglycan) serves as a novel suppressor of cancer progression in epithelial tumors; however, its role in hematologic malignancies is unknown. Here we demonstrate that TβRIII protein expression is decreased or lost in the majority of human multiple myeloma specimens. Functionally, restoring TβRIII expression in myeloma cells significantly inhibited cell growth, proliferation, and motility, largely independent of its ligand presentation role. In a reciprocal fashion, shRNA-mediated silencing of endogenous TβRIII expression enhanced cell growth, proliferation, and motility. Although apoptosis was not affected, TβRIII inhibited proliferation through induction of the cyclin-dependent kinase inhibitors p21 and p27. TβRIII further regulated myeloma cell adhesion, increasing homotypic myeloma cell adhesion while decreasing myeloma heterotopic adhesion to bone marrow stromal cells. Mechanistically, live cell imaging of myeloma and stroma cell cocultures revealed that TβRIII-mediated inhibition of heterotrophic adhesion was associated with decreased duration of myeloma/bone marrow stromal cell interaction. These results suggest that loss of TβRIII expression during multiple myeloma progression contributes to disease progression through its functional effects on increased cell growth, proliferation, motility, and adhesion.

INTRODUCTION

Multiple myeloma is the second most common hematologic malignancy in the United States, accounting for 1–2% of all cancers and 10–15% of hematologic tumors (Jemal et al., 2007). Despite advances in conventional and high-dose therapy, multiple myeloma remains incurable with a 5-yr survival of 31% (Brenner, 2002). Lack of insight into the mechanisms of chemotherapeutic resistance remains a significant barrier to treatment of this disease. Myeloma cells localize in the bone marrow, where their survival is dependent on the normal stromal cells that secrete cytokines and interact with malignant cells through adhesion molecules (De Vos and Klein, 2004). Several growth factors—including interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), and transforming growth factor-β (TGF-β)—play an important role in multiple myeloma pathogenesis and mediate tumor cell proliferation, drug resistance, and migration in the bone marrow milieu (Yasui et al., 2006). A better understanding of how these signaling pathways are functionally linked is required to design therapeutic strategies that target the myeloma cell in the bone marrow microenvironment. The TGF-β signaling pathway plays an important role in regulating normal hematopoiesis, inhibiting proliferation while stimulating differentiation when appropriate (Dong and Blobe, 2006). TGF-β
ligands regulate cellular processes by binding to three high-affinity cell surface receptors: the type I TGF-β receptor (TβRI), the type II TGF-β receptor (TβRII), and the type III TGF-β receptor (TβRIII). TβRII, the most abundant TGF-β receptor, has traditionally been thought to function as a coreceptor, enhancing ligand binding to associated receptors (Kirkbride et al., 2005, 2008). Substantial evidence, however, supports an essential role for TβRII, including the embryonic lethal phenotype of TβRII knockout mice, in part due to ineffective erythropoiesis (Stenvers et al., 2003), and the essential and nonredundant role for TβRII in the endothelial-to-mesenchymal transition of chick heart development (Brown et al., 1999). TβRII has been demonstrated to serve as a suppressor of cancer progression in cancers of the breast, kidney, lung, ovary, pancreas, and prostate (Dong et al., 2007; Hempel et al., 2007; Turley et al., 2007; Finger et al., 2008; Gordon et al., 2008; Margulis et al., 2008). Like other coreceptors, TβRII undergoes ectoderm shedding, releasing a soluble form of TβRII (sTβRII), which has the potential to serve as an antagonist of TβRII signaling (Lopez-Casillas et al., 1994). Moreover, work in our laboratory has established a role for sTβRII in decreasing cancer cell migration and invasion (Dong et al., 2007). The mechanisms by which TβRII and sTβRII receptors regulate TGF-β superfamily signaling remain poorly understood.

TGF-β normally functions to suppress the proliferation and immunoglobulin (Ig) production of B cells (Kehrl et al., 1986). In multiple myeloma, resistance to the homeostatic functions of TGF-β signaling develops, perhaps through defective trafficking of TβRI and TβRII to the cell surface (Amoroso et al., 1998; Fernandez et al., 2002). In response, both human myeloma and bone marrow stromal cells (BMSCs) from myeloma patients secrete higher levels of TGF-β compared with normal plasma cells (Urashima et al., 1996; Hayashi et al., 2004), contributing to the immune dysfunction present in multiple myeloma patients (Hayashi et al., 2004). Importantly, TGF-β-neutralizing antibodies or a TβRI inhibitor can block IL-6 and VEGF secretion and decrease myeloma cell growth and cell adhesion to the BMSCs (Urashima et al., 1996; Hayashi et al., 2004). These results establish resistance of myeloma cells to the homeostatic effects of TGF-β and its effect on the bone marrow stroma as important factors in the pathogenesis of multiple myeloma. Here we investigate the role of TβRII in multiple myeloma.

RESULTS

TβRII expression is decreased in human multiple myeloma

TβRII expression is decreased or lost in several human epithelial tumors, including cancers of the breast, lung, ovary, and prostate (Dong et al., 2007; Hempel et al., 2007; Turley et al., 2007; Finger et al., 2008), with loss of expression correlating to disease progression and predicting a poorer prognosis for patients. Functionally, loss of TβRII results in increased motility and invasion in vitro and increased tumorigenicity, angiogenesis, and invasiveness in vivo (Dong et al., 2007; Hempel et al., 2007; Turley et al., 2007; Finger et al., 2008). To investigate TβRII expression during multiple myeloma progression, we initially examined genomic data available through the publicly available Oncomine Cancer Profiling Database. TβRII mRNA expression was decreased during multiple myeloma progression, with decreased expression in bone marrow specimens from patients with monoclonal gammopathy of undetermined significance (MGUS) relative to normal patients, and was decreased in bone marrow from multiple myeloma patients relative to bone marrow from MGUS patients (Mattioi et al., 2005; Zhan et al., 2006) (Supplemental Figure S1, summarized in Supplemental Table S1). In contrast, there was little to no change in the expression of the other major TGF-β receptors examined, including TβRII, TβRI (ALK-5), endoglin, and ALK-1 (unpublished data).

We previously demonstrated that TβRIII protein expression is largely regulated at the message level and that altering levels of TβRIII protein expression was sufficient to regulate TGF-β signaling (Blobe et al., 2001; Chen et al., 2003). To examine TβRIII protein expression in multiple myeloma, we performed immunohistochemistry (IHC) analysis on human bone marrow biopsy specimens from multiple myeloma patients. Using a tissue microarray (US Biomax, Rockville, MD) containing 10 cases of myeloma, 11 normal tissue controls, and an anti-TβRIII specific antibody as previously reported (Dong et al., 2007), we established that TβRIII expression was decreased in multiple myeloma specimens compared with normal controls (Figure 1, A–C). TβRIII expression decreased by 60% when comparing normal controls to multiple myeloma specimens (p < 0.0001, Figure 1B). When examining TβRIII expression based on level of expression (low IHC score = 0–1, medium IHC score = 2–3, high IHC score = 4–5), the percentage of samples with high TβRIII expression decreased from 81% of normal controls to 15% of multiple myeloma specimens (p < 0.0001), whereas the percentage of samples with low TβRIII expression increased from 0% of normal controls to 40% of multiple myeloma specimens (p < 0.001, Figure 1C). In addition, three of four myeloma cell lines demonstrated decreased/lost TβRIII expression at the mRNA (unpublished data) and protein level (Figure 1D).

TβRII inhibits cell growth and proliferation in multiple myeloma cells

We previously demonstrated that TβRIII enhanced TGF-β1–mediated growth inhibition in L6 myoblasts and that the cytoplasmic domain of TβRIII contributed to this response (Blobe et al., 2001; Chen et al., 2003; You et al., 2007), whereas TβRII had little effect on the proliferation of breast, lung, or prostate cancer cells. To investigate the role of TβRIII in non-epithelium-derived cancer cells, we transiently expressed TβRII in the RPMI-8226 myeloma cell line, which lacks endogenous TβRIII expression (Figure 1D), using TβRIII-expressing adenovirus tagged with green fluorescent protein (GFP) or GFP-expressing adenovirus alone as a control (Figure 2A). Here and throughout, infected RPMI-8226 cells were sorted and gated on GFP, and the adenovirally infected cells were used. Restoring expression of TβRII in RPMI-8226 cells resulted in a 50–60% reduction in cell growth (Figure 2B; p < 0.05) and a 40–50% reduction in proliferation (Figure 2C; p < 0.001) relative to control cells. We next investigated the effect of shRNA-mediated silencing of endogenous TβRIII expression in the U266 myeloma cell line (Figure 1D), with a nontargeting vector control (NTC) shRNA as control (Figure 2A). Here and throughout, U266 cells were sorted and gated on dsRED, and the adenovirally infected cells were used. Reducing TβRIII expression in U266 cells increased cell growth by 60% (Figure 2D; p < 0.05) and increased proliferation by 75% (Figure 2E; p < 0.001) relative to control. These data indicate that TβRIII inhibits multiple myeloma cell growth and proliferation.

TβRIII serves as a coreceptor for multiple TGF-β superfamily ligands, including TGF-β and bone morphogenetic proteins (BMPs) (Kirkbride et al., 2008). To determine whether TβRIII-mediated growth inhibition of myeloma cells was ligand-dependent, we examined the effect of several TGF-β superfamily ligands (BMP-2, BMP-4, TGF-β1) on cell growth and inhibition in the presence or absence of TβRII. Whereas BMP-2, BMP-4, and TGF-β1 each modestly inhibited myeloma cell proliferation, in each case TβRIII inhibited proliferation to an equivalent extent in the presence and absence of ligand (Supplemental Figure S2). We further examined the
RIII regulates proliferation, we examined the effect of TβRIII on multiple myeloma cell growth and proliferation of multiple myeloma cells to BMSCs. We examined the effect of shRNA-mediated silencing of endogenous TβRIII on adhesion of multiple myeloma cells to BMSCs, we examined the effect of silencing of endogenous TβRIII by U266 myeloma cell line, with an NTC shRNA as control. Reducing TβRIII expression in U266 cells increased adhesion to HS-5 stromal cells by 50–50% (Figure 3). To determine the contributions of specific domains of TβRIII to adhesion, we transiently expressed TβRIIIΔGAG (lacking the glycosaminoglycan chains), TβRIIIΔCYTO (lacking the cytoplasmic domain) (Figure 4B), or GFP alone as control in RPMI-8226 cells and cocultured these with HS-5 stromal cells. Both TβRIII and TβRIIIΔGAG significantly decreased adhesion of multiple myeloma cells to HS-5 stromal cells by 45–60% (p < 0.05) and 30–50% (p < 0.05), respectively (Figure 4B). Interestingly, TβRIIIΔCYTO did not significantly decrease adhesion compared with control cells (Figure 4B). These results suggest that TβRIII requires its cytoplasmic domain for its inhibitory function on heterotopic adhesion.

To explore the mechanism by which TβRIII functions to inhibit adhesion of myeloma cells to BMSCs (HS-5), we tracked the movement of individual RPMI-8226-TβRIII-GFP or RPMI-8226 GFP control adenovirally infected myeloma cells when cocultured with HS-5 BMSCs using live cell imaging. We observed that the TβRIII-expressing myeloma cells appeared to interact more often but less efficiently with the BMSCs compared with control myeloma cells/BMSCs. To quantify these observations, we determined both the number of myeloma cell/BMSC interactions and the amount of time that the myeloma/BMSCs were interacting. We determined the number of TβRIII-infected multiple myeloma cells that were interacting with HS-5 BMSCs as defined by >50% multiple myeloma cell body buried underneath the BMSC body at least one time during the period of observation (Figure 4C; Supplemental Video 1) and found that 60% of total TβRIII-expressing myeloma cells initiated this contact with HS-5 BMSCs compared with 27% of GFP control myeloma cells (Figure 4C; p < 0.05). TβRIII-expressing multiple myeloma cells,
however, spent less time (30 min for TβRIII vs. 36 min for GFP) within a 15 μm perimeter of the BMSCs (Supplemental Video 2: Figure 4D), although this was not statistically significant. Importantly, TβRIII-expressing multiple myeloma cells spent approximately threefold less time compared with GFP control myeloma cells (8 min for TβRIII and 22 min for GFP) interacting with the HS-5 BMSCs when they were underneath the stromal cells compared with control cells (Figure 4E; p < 0.05). Taken together, these data indicate that whereas TβRIII-expressing multiple myeloma cells are proficient in establishing initial contact with the BMSCs, TβRIII-infected multiple myeloma cells were unable to sustain these interactions with BMSCs, resulting in inhibition of heterotropic cell–cell adhesion. These data collectively indicate that loss of TβRIII may contribute to the pathogenesis of multiple myeloma by enhancing adhesion and localization of multiple myeloma cells to BMSCs.

**TβRII increases homotypic aggregation of multiple myeloma cells**

One potential mechanism for decreased heterotropic cell–cell adhesion to stromal cells could be increased homotypic myeloma cell–cell adhesion. To determine the effects of TβRII on homotypic aggregation of myeloma cells, we transiently expressed TβRII, TβRIIΔGAG, TβRIIΔCYTO, or GFP alone as control in RPMI-8226 multiple myeloma cells and quantified the number of aggregates at different time points. TβRII expression significantly increased the number of aggregates at all time points (15–120 min) relative to control cells (Figure 5A; p < 0.05). Interestingly, neither TβRIIΔGAG nor TβRIIΔCYTO expression increased homotypic aggregation in comparison to control cells (Figure 5A). These data indicate that
TβRIII is important in mediating homotypic cell–cell adhesion in myeloma cells and, structurally, both the cytoplasmic domain and glycosaminoglycan chains are important in mediating this effect. We next investigated the effect of shRNA-mediated silencing of TβRIII expression in the U266 myeloma cell line. Reducing TβRIII expression in U266 cells significantly decreased homotypic aggregation (Figure 5B; p < 0.05) relative to control cells. These data indicate that TβRIII directly promotes homotypic cell–cell adhesion of myeloma cells.

**TβRIII decreases migration/chemotaxis of multiple myeloma cells**

Increasing or restoring TβRIII expression in cancer models decreases cancer cell motility and invasion in vitro and angiogenesis, invasion, and metastasis in vivo (Dong et al., 2007; Hempel et al., 2007; Finger et al., 2008; Gordon et al., 2008; Mythreye and Blobe, 2009). To determine whether TβRIII-mediated suppression of migration also occurs in a multiple myeloma cell model system, we examined transwell migration of multiple myeloma cells toward serum (10% fetal bovine serum [FBS], chemotactic gradient). We transiently expressed TβRIII in RPMI-8226 cells using TβRIII-expressing adenovirus tagged with GFP or GFP-expressing adenovirus alone as a control. Expression of TβRIII resulted in a >80% reduction in chemotactic migration relative to control cells (Figure 6A; p < 0.0001). To determine the structural requirements for TβRIII inhibition of migration, we expressed as graphs using the Microsoft Excel program. The percentage of infected myeloma cells that interacted with the BMSCs was calculated as the percentage of total myeloma cells that were at least 50% underneath the HS-5 BMSC, at least once during the period of observation (C) and is represented graphically (*p < 0.05). The time that the infected myeloma cells spent interacting with the BMSCs was calculated as both time (minutes) that the myeloma cells spent within a 15 μm perimeter of the HS-5 BMSCs (D) and the time (minutes) that the myeloma cells spent interacting with HS-5 BMSC before exiting (E) and is represented graphically (*p < 0.05). Representative pictures above C and D show myeloma/stromal cell interactions (original magnification, 60×). For the live cell–imaging analysis, at least 35 cells were counted for each experiment during the 120-min time period. All values represent means ± SEM and are representative of at least two independent experiments.
also transiently expressed TβRIIIΔGAG and TβRIIIΔCYTO, and assessed their ability to regulate migration relative to full-length TβRIII. In comparison to full-length TβRIII, which significantly suppressed migration by >80% (p < 0.0001), TβRIIIΔGAG and TβRIIIΔCYTO suppressed migration by ~60% (p < 0.0001) and 50% (p < 0.0001) in RPMI-8226 cells, respectively (Figure 6A), suggesting a requirement for both the cytoplasmic domain and the glycosaminoglycan chains in mediating this effect. We next investigated the effect of shRNA-mediated silencing of endogenous TβRIII expression in U266 myeloma cell line. Reducing TβRIII expression in U266 cells increased cell migration by ~80% (Figure 6B; p < 0.05). Taken together, these data indicate that TβRIII directly inhibits the ability of myeloma cells to migrate.

To address the extent to which TβRIII regulates migration independent of TGF-β superfamily signaling, we next examined the effects of TβRIII on migration in the context of the TGF-β superfamily ligands, BMP-2 and TGF-β1, as well as in the presence of the small molecule TβRII/ALK-5 inhibitor. Although BMP-2, TGF-β1, and the small molecule TβRII/ALK-5 inhibitor all inhibited myeloma cell migration, in comparison to GFP-control RPMI-8226 myeloma cells, TβRIII-expressing myeloma cells significantly inhibited migration in the presence or absence of BMP-2, TGF-β1, or the small molecule TβRII/ALK-5 inhibitor (Figure 6C). These data suggest that TβRIII largely inhibits migration independent of its ligand presentation role.

**DISCUSSION**

The TβRIII coreceptor has a well-established role as a suppressor of cancer progression in a broad spectrum of epithelium-derived tumors (Dong et al., 2007; Hempel et al., 2007; Turley et al., 2007; Finger et al., 2008; Gordon et al., 2008; Margulis et al., 2008). Although the TGF-β signaling pathway has defined roles in multiple myeloma disease pathogenesis, little is known about the mechanisms that result in resistance of myeloma cells to the homeostatic functions of TGF-β. Here we investigated the role of TβRIII in multiple myeloma disease progression, demonstrating that TβRIII expression is decreased during disease progression at both the mRNA and protein level. Although the control bone marrow had a low number of plasma cells, we identified these cells with a certified hematopathologist and established that the plasma cells in normal patient specimens contained high TβRIII expression whereas plasma cell–derived myeloma cells had low TβRIII expression. TβRIII expression was also decreased/lost at the mRNA and protein level in the majority of human multiple myeloma cell lines analyzed. Thus, loss of TβRIII expression is the most frequent alteration in the TGF-β signaling pathway in multiple myeloma described to date. Importantly, restoring TβRIII expression decreased cell growth, proliferation, heterotopic adhesion, and migration in myeloma cells, whereas silencing of endogenous TβRIII expression increased cell growth, proliferation, heterotopic adhesion, and migration. On the basis of our findings, we believe that loss of TβRIII may contribute to the pathogenesis of multiple myeloma by promoting increased multiple myeloma cell growth, proliferation, migration, and adhesion to BMSCs.

How might the loss of TβRIII expression result in increased proliferation in multiple myeloma? As in other human cancers, resistance to TGF-β-mediated growth inhibition is a common event in multiple myeloma. The mechanisms of resistance to TGF-β-mediated growth inhibition in myeloma are unknown, however. In epithelial cancers, resistance to TGF-β-mediated growth inhibition may occur through reduced expression of TGF-β receptors or their signal transducers (Roberts and Wakefield, 2003; Hempel et al., 2008). Based on the current data, the increased proliferation of myeloma cells could be due, at least partly, to decreased/lost TβRIII expression. Mechanistically, the antiproliferative effects of the TGF-β signaling pathway are
mediated through regulation of the cdk proteins that drive the G1 phase of the cell cycle, namely cdk2, cdk4, and cdk6 (Massague et al., 2000). In epithelial cells from skin, lung, and breast, TGF-β increases the cdk4/cdk6 inhibitor, p15Ink4b, and the cdk2 inhibitor, p27Kip1 (Hannon and Beach, 1994; Warner et al., 1999). In keratinocytes and in colon and epithelial cells, TGF-β also increases a p27-related inhibitor, p21Cip1 (Elbendary et al., 1994; Datto et al., 1995; Li et al., 1995). Here we demonstrate that TβRIII increased expression of the cdk inhibitors, p21 and p27, and that this effect was enhanced by the TGF-β ligands, TGF-β1, BMP-2, and BMP-4. In contrast, TβRIII did not induce apoptosis in myeloma cells. Thus, TβRIII appears to regulate myeloma cell proliferation through induction of p21 and p27. Although mechanisms for TGF-β-induced transcription of p21 and p27 have largely been established, the mechanisms by which TβRIII regulates their expression are currently under investigation.

The effects of TβRIII on multiple myeloma cell adhesion are quite intriguing, particularly as multiple myeloma pathogenesis is dependent on the interaction of multiple myeloma cells with the stroma. Binding of multiple myeloma cells to BMSCs triggers expression of adhesion molecules and secretion of cytokines that are known to promote myeloma cell growth, survival, drug resistance, and migration (Teoh and Anderson, 1997). Here we demonstrate that TβRIII decreased multiple myeloma cell adhesion to BMSCs, while increasing homotypic cell–cell adhesion. These effects are specific, as we also investigated the effect of TβRIII expression on adhesion of multiple myeloma cells to extracellular matrix proteins, fibronectin, laminin, and collagen and did not find any significant difference between TβRIII-expressing multiple myeloma cells versus control multiple myeloma cells (unpublished data). How might TβRIII specifically mediate differential effects on heterotypic and homotypic adhesion? One straightforward explanation is that increased homotypic interactions would leave less surface area available for interaction with stromal cells. This may be the case, but by using live cell imaging to track the behavior of individual cells, we demonstrated that TβRIII increases the initial interaction of multiple myeloma cells with BMSCs. The durability of this interaction is decreased, however, relative to multiple myeloma cells in the absence of TβRIII. These results suggest that, although TβRIII may be functioning, in part, as an adhesion receptor, upon multiple myeloma–stroma contact, additional adhesion receptor interactions and/or signaling may regulate the duration of this interaction. A role of TβRIII-mediated signaling in regulating multiple myeloma cell adhesion is further supported by the dependence of the TβRIII cytoplasmic domain for both heterotypic and homotypic adhesion. The importance of myeloma cell–stromal cell adhesion for survival and for resistance to chemotherapeutic drugs is well documented (Uchiyama et al., 1993; Hazlehurst et al., 2000), suggesting that treatments designed to restore TβRIII expression/function in multiple myeloma may represent an attractive therapeutic target.

FIGURE 6: TβRIII decreases migration/chemotaxis of human multiple myeloma cells to BMSCs. (A) RPMI-8226 myeloma cells were adenovirally infected with GFP vector, GFP-TβRIII, GFP-TβRIII-cyto, or GFP-TβRIII-GAG and incubated for 48 h. Cells were resuspended in SFM, and $5 \times 10^5$ cells in 200 μl were placed in the top of the transwell chamber and allowed to migrate to the bottom chamber (containing 700 μl of 10% FBS-supplemented medium) for $> 12$ h ($\ast \ast p < 0.001$). All values represent means ± SEM in triplicate cultures and are representative of three independent experiments. (C) RPMI-8226 myeloma cells were adenovirally infected with GFP vector or GFP-TβRIII. After 48 h of infection, cells were resuspended in SFM, and $5 \times 10^5$ cells in 200 μl were placed in the top of the transwell chamber and treated with vehicle control, 10 nM BMP-2, 200 pM TGF-β1, or 5 μM ALK5 inhibitor. Migration toward 700 μl of 10% FBS-supplemented medium was allowed for $12$ h, and the myeloma cells that had migrated to the bottom chamber were subsequently counted ($p < 0.05$, $\ast \ast p < 0.001$).
Cell migration involves dynamic regulation of adhesion events. We have previously demonstrated in several epithelial tumors that TβRIII inhibits cancer cell migration, invasion, and metastasis in vitro and in vivo (Dong et al., 2007; Hempel et al., 2007; Turley et al., 2007; Finger et al., 2008; Gordon et al., 2008). Mechanistically, we have demonstrated that TβRIII-mediated inhibition of migration occurs through β-arrestin2–mediated Cdc42 activation, which alters actin cytoskeleton organization to decrease directional persistence (Mythreye and Blobe, 2009). Furthermore, TβRIII inhibits both random and chemotactic migration, and was independent of canonical TGF-β signaling (Mythreye and Blobe, 2009). Unlike most epithelial tumors, however, which have the propensity to metastasize to distant organs, multiple myeloma malignant plasma cells often home to their native bone marrow, where they are dependent on interactions within their environment. In the present study, we demonstrate that TβRIII has inhibitory effects on chemotactic multiple myeloma cell migration. Mechanistically, the TβRIII–mediated inhibition was at least partially dependent on its cytoplasmic and glycosaminoglycan chain domains. The relative contribution of TβRIII’s effects on adhesion and on signaling to Cdc42 and the actin cytoskeleton on multiple myeloma cell migration are currently under investigation.

The genomic locus for TβRIII, TGFBR3, is on the short arm of chromosome 1, 1p32, a region that is frequently deleted in human epithelium-derived cancers, including cancers of the breast, colon, endometrium, kidney, lung, ovary, stomach, and testis (Johnson et al., 1995; Ragnarsson et al., 1999). We have demonstrated loss of heterozygosity at the TGFBR3 locus in 37–50% of breast cancer, non–small cell lung cancer, and prostate cancer patients (Dong et al., 2007; Turley et al., 2007; Finger et al., 2008). Additionally, the short arm of chromosome 1 has been reported to be lost in multiple myeloma patients (Taniwaki et al., 1994; Shaughnessy et al., 2007). We have also previously demonstrated that loss of TβRIII expression in human cancers could occur through epigenetic regulation or TGF-β1–mediated down-regulation of TGFBR3 at the transcriptional level (Dong et al., 2007; Hempel et al., 2007; Turley et al., 2007; Finger et al., 2008; Hempel et al., 2008). As multiple myeloma patients have higher concentrations of TGF-β1, this increase may represent another mechanism for suppression of TβRIII expression. The mechanism by which TβRIII expression is decreased and whether TβRIII is a tumor suppressor gene on chromosome 1p32 in multiple myeloma remain to be defined.

In summary, the current findings define a novel role for TβRIII in the pathophysiology of multiple myeloma as an inhibitor of proliferation, heterotrophic cell–cell adhesion, and migration, all largely independent of its ligand presentation role. These results provide a model to study myeloma plasma cell dissemination within the bone marrow microenvironment. Whether TβRIII plays a similar role in other hematologic malignancies and whether therapies designed to restore TβRIII function could circumvent chemotherapeutic resistance remain to be explored.

**MATERIALS AND METHODS**

**Cell culture and reagents**

Human multiple myeloma cell lines NCI-H929, OPM2, RPMI-8226, and U266 and human BMSC line HS-5 were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were maintained in RPMI-1640 medium supplemented with 10% FBS. The cells were grown in 5% CO2 at 37°C in a humidified atmosphere. In all assays, RPMI-8226 myeloma cells were adenovirally infected with GFP vector or GFP-TβRIII and incubated for 48 h. U266 myeloma cells were adenovirally infected with NTC or shRNA-TβRIII and incubated for 72 h before initiation of the assay. In all experiments, infected RPMI-8226 and U266 cells were sorted, gating on GFP or dsRED, using a FACSARia cell sorter (BD Biosciences, Franklin Lakes, NJ), and the adenovirally infected cells were used.

**TβRIII protein analysis on bone marrow tissue array**

Immunohistochemical studies were done on a paraffin-embedded tissue microarray containing myeloma and normal tissue controls with duplicate cores per case (US Biomax, Rockville, MD). Tissues were probed with a purified anti-human TβRIII-specific polyclonal antibody. Immunoreactivity and specificity of this antibody for TβRIII has been previously verified in immunohistochemical studies in our laboratory (Dong et al., 2007). Following rehydration and blocking with 1% hydrogen peroxide, 10% goat serum/phosphate-buffered saline (PBS), and an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA), tissue samples were incubated overnight at 4°C with TβRIII-specific antibody or preimmune serum at a dilution of 1:200–400 (vol:vol) in 10% goat serum/PBS. Following secondary antibody incubation with biotinylated anti-rabbit IgG at room temperature for 1 h, tissues were incubated with Vectastain ABC reagent (Vector) for 30 min, and immunoreactivity was visualized using the avidin-biotin complex immunoperoxidase system and diaminobenzidine (Vector). Slides were counterstained with hematoxylin, and immunoreactivity for TβRIII in the specimen was scored by staining intensity in a blinded manner with 0–1 = no or weak staining, 2–3 = moderate staining, and 4–5 = intense staining. Standards for each staining score were used to maintain consistent scoring across specimens.

**TGF-β binding and cross-linking assay**

Cells were incubated with Krebs-Ringer-HEPES (KRH) buffer (50 mM HEPES, pH 7.5, 130 mM NaCl, 5 mM MgSO4, 1 mM CaCl2, and 5 mM KCl) containing 0.5% bovine serum albumin (BSA) for 30 min at 37°C, and then with 100 pM 125I-TGFβ1 for 3 h at 4°C. 125I-TGFβ1 was cross-linked with 0.5 mg/ml disuccinimidyl suberate for 15 min and quenched with 20 mM glycine for 10 min. Cells were then washed with KRH buffer, lysed in RIPA buffer, and immunoprecipitated at 4°C overnight using a polyclonal antibody directed toward the extracellular domain of TβRIII (R&D Systems, Minneapolis, MN) bound to protein G sepharose beads. All samples were analyzed by SDS–PAGE and phosphorimaging analysis of dried gels.

**Adenoviral constructs**

All adenoviral constructs were made using the Becton Dickinson Adeno-X expression system (BD Biosciences), purified using the Adeno-X Virus Purification Kit and titered using the Adeno-X Rapid Titer Kit. The TβRIII and NTC shRNA sequences were generated by Dharmaco (Lafayette, CO).

**Cell growth**

Cells were plated at 1.0 × 104 cells/ml in 24-well plates. Cell growth was measured by trypan blue exclusion.

**[3H]Thymidine incorporation assay**

Cell sorting was done on the adenovirally infected myeloma cell lines by gating the GFP- and dsRED-expressing cells and subtracting out the uninfected cells. Adenovirally infected GFP- or dsRED-expressing cells were then concentrated using a FACSARia cell sorter (BD Biosciences) and plated at 1.0 × 104 cells/ml in 96-well, flat-bottom culture plates to a final volume of 200 μl. After incubation for 24 h, cultures were pulsed with 1 μCi [3H]thymidine (Amersham Biosciences, Piscataway, NJ) for 4 h, harvested, and counted in a
MicroBeta Trilux liquid scintillation counter (EG&G Wallac, Turku, Finland) in triplicate.

Apoptosis analysis
Dual staining with allophycocyanin conjugate (APC) annexin V and 7-aminoactinomycin D (7-AAD) was used to detect apoptosis. Flow cytometric analysis was performed using FACScan (BD Biosciences). Myeloma cells were treated with or without 50 nM bortezomib (IC50 determined by MTT assay) for 6 h as a positive control for apoptosis. Cells were then washed with PBS and stained with APC annexin V (AN) and 7-AAD according to the manufacturer's protocol (catalogue #550475; BD Biosciences, San Jose, CA). Ten thousand cells were analyzed by flow cytometry and gated for GFP+ or dsRED+ expressing cells, and then AN−/7-AAD−, AN+/7-AAD−, and AN+/7-AAD+ populations were enumerated, which correspond to live cells, early apoptotic cells, and both late apoptotic and necrotic cells, respectively. Data were analyzed using FlowJo software.

Immunoblot analysis
Myeloma cells were treated with TGF-β superfamily ligands, BMP-2 (0–10 nM), BMP-4 (0–10 nM), TGF-β1 (0–200 pM), or vehicle for the indicated time points. In the apoptosis experiment, cells were treated with or without bortezomib as described in the previous section. Lysates were harvested with hot sample buffer and boiled for 5 min. Lysates were separated using 10% SDS–PAGE and transferred onto polyvinylidene difluoride membranes. Western blotting was performed using the α-p21, p27, phospho-Smad2, total Smad 2, phospho-Smad1/5, and total Smad1/5 antibodies (Cell Signaling Technology, Danvers, MA). Western blotting for TβRIII expression was performed using an antibody directed toward the extracellular domain of TβRIII (R&D Systems) and an α-β-actin antibody (Sigma, St. Louis, MO) to control for protein loading.

Adhesion assay
HS-5 cells were plated at a density of 10–20 × 10³ per well in black, 96-microwell, flat-bottom polystyrene plates and allowed to adhere for 24 h. The indicated adenovirally infected multiple myeloma cell lines RPMI-8226 and U266 (1–2 × 10³) were washed three times with PBS, resuspended in serum-free RPMI medium (SFM) with 5 μM calcine-blue AM (BD Biosciences) for 30 min at 37°C and 5% CO₂. The cells were then washed in PBS, resuspended in SFM + 1% BSA, and plated onto the BMSC-coated, 96-well plates (HS-5) for 2 h. Nonadherent myeloma cells were removed by carefully washing twice with SFM and inverting the plates. SFM (200 μl) was then added to each well. Adherent cells were quantified in a fluorescence multimwell plate reader (Wallac Victor2). Triplicate cultures were set up for every cell population tested.

Live cell imaging
For live cell imaging of the coculture experiments, HS-5 cells were plated on 35-mm glass-bottom dishes (MatTek, Ashland, MA) at a density of 10⁵ cells per well in regular culture medium and allowed to adhere for 24 h. Cells were then washed twice in PBS. The indicated adenovirally infected (GFP vector or GFP-TβRIII) RPMI-8226 multiple myeloma cells suspended in SFM + 1% BSA (5 × 10³) were then plated onto the BMSC-coated plates (HS-5) and placed in a temperature- and CO₂-controlled chamber. A Zeiss (Thornwood, NY) Axio Observer Z1 motorized microscope equipped with 20, 40, and 100x objective lenses was used for the imaging. Time-lapse recordings were started ~10 min after cells were cocultured with infected RPMI-8226 myeloma cells. The images were collected at 1-min intervals for >120 min with a cooled charge-coupled device (CCD) video camera (Coolsnap ES high resolution CCD camera) operated by a Metamorph image analysis software (Molecular Devices, Sunnyvale, CA). The adhesion parameters, including percent total cells and time (in minutes) that the infected RPMI-8226 myeloma cells interacted with the HS-5 BMSCs, were obtained from time-lapse movies. Cells were manually traced for each cell using Metamorph to track the adhesion characteristics of the individual cells. The percentage of infected myeloma cells that interacted with the BMSCs was calculated as the percentage of total myeloma cells that were at least 50% in contact or buried underneath the HS-5 BMSC. The time that the infected myeloma cells spent interacting with the BMSCs was calculated as both time (minutes) that the myeloma cells spent within a 15 μm perimeter of the HS-5 BMSCs and the time (minutes) that the myeloma cells spent in contact (>50%) with HS-5 BMSCs before exiting. At least 35 cells were counted for each experiment during the 120-min time period.

Aggregation assay
RPMI-8226 multiple myeloma cells were adenovirally infected with GFP vector, GFP-TβRIII, GFP-TβRIIIΔACYTO (mutant lacking the cytoplasmic domain), and GFP-TβRIIIΔAGAG (mutant lacking glycosaminoglycan chains). After 48 h of incubation at 37°C and 5% CO₂, the cells were placed into a 96-microwell plate with RPMI 1640 medium supplemented with 10% FBS. U266 cells were adenovirally infected with NTC or shRNA-TβRIII and incubated for 72 h. After the myeloma cells were dispersed with a pipette, aggregation was observed at the indicated time points. The number of aggregates in clumps of ≥3, ≥5, and ≥10 were counted. Triplicate cultures were set up for every cell population tested.

Migration assay
The indicated adenovirally infected RPMI-8226 and U266 myeloma cells were washed three times with PBS and resuspended in SFM at a density of 5 × 10⁶ cells in 200 μl in the upper chamber of a transwell filter (8 μm pore transwell insert; Costar, Cambridge, MA) to assess cell migration/chemotaxis. As indicated, cells were treated with either no ligand, 10 nM BMP-2, 200 pM TGF-β1, or 5 μM ALK5 inhibitor. Cells were allowed to migrate for 12 h at 37°C toward the lower chamber containing 700 μl of RPMI 1640 medium with 10% FBS. Cells that migrated to the bottom chamber were then counted by trypan blue exclusion. Duplicate cultures were set up for every cell population tested.

Statistical analysis
Significance of results was assessed using Student's t test for paired samples; *p < 0.05, **p < 0.001, ***p < 0.0001. Error bars, where indicated, represent SEM (n = 3).

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