Acute Tissue Injury Activates Satellite Cells and Promotes Sarcoma Formation via the HGF/c-MET Signaling Pathway

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Abstract

Some patients with soft-tissue sarcoma (STS) report a history of injury at the site of their tumor. Although this phenomenon is widely reported, there are relatively few experimental systems that have directly assessed the role of injury in sarcoma formation. We recently described a mouse model of STS whereby p53 is deleted and oncogenic Kras is activated in muscle satellite cells via a Pax7\(^{creER}\) driver following intraperitoneal injection with tamoxifen. Here, we report that after systemic injection of tamoxifen, the vast majority of Pax7-expressing cells remain quiescent despite mutation of p53 and Kras. The fate of these muscle progenitors is dramatically altered by tissue injury, which leads to faster kinetics of sarcoma formation. In adult muscle, quiescent satellite cells will transition into an active state in response to hepatocyte growth factor (HGF). We show that modulating satellite cell quiescence via intramuscular injection of HGF increases the penetrance of sarcoma formation at the site of injection, which is dependent on its cognate receptor c-MET. Unexpectedly, the tumor-promoting effect of tissue injury also requires c-Met. These results reveal a mechanism by which HGF/c-MET signaling promotes tumor formation after tissue injury in a mouse model of primary STS, and they may explain why some patients develop a STS at the site of injury. Cancer Res. 75(3): 605–14. ©2014 AACR.

Introduction

Soft-tissue sarcomas (STS) represent a heterogeneous mix of >50 mesenchymal tumor types that together encompass approximately 1% of tumors in adults and 9% of tumors in children (1–3). To study sarcoma development, we utilized Pax7\(^{creER}\)/; Kras\(^{-12D\_G12D}\)/; Trp53\(^{fl/fl}\) (P7KP) mice because mutations in the Ras and p53 pathways have been reported in human STS (4, 5). Following systemic administration of tamoxifen by intraperitoneal injection, P7KP mice develop sarcomas throughout the animal in 6 to 8 weeks (6). The most common locations for sarcoma development are the body wall, extremities, and head and neck (6). Similar to other reports, the histology of the sarcomas in P7KP mice exists along a continuum of undifferentiated pleomorphic sarcoma (UPS), myogenic UPS, and embryonal rhabdomyosarcoma (6, 7). To develop a temporally and spatially restricted model of STS, we injected P7KP mice with 4-hydroxytamoxifen (4OHT) directly into the gastrocnemius muscle. Remarkably, sarcomas developed at the 4OHT injection site with 100% penetrance with a median time that was approximately twice as fast as when the P7KP mice received systemic tamoxifen, which prompted us to test the hypothesis that more rapid sarcoma formation was caused by tissue injury related to 4OHT administration.

Although others have reported an association between injury and sarcoma development (8–14), P7KP mice represent a unique model system to investigate the mechanism. P7KP mice are a mammalian system in which the timing of p53 loss and Kras activation is tightly controlled in a defined population of cells (muscle satellite cells). Our experiments demonstrate that the fate of Pax7\(^{+}\) cells harboring oncogenic mutations is altered in the setting of muscle injury in a process dependent on hepatocyte growth factor (HGF)/c-MET signaling. HGF/c-MET signaling plays an important role in regulating the proliferation of muscle progenitors following injury (15), and we show that it is also required for rapid sarcoma formation in our model system. Therefore, we propose that the activation state of the sarcoma cell of origin serves as a barrier to tumor formation. This tumor suppressor mechanism may explain why sarcomas are relatively rare.

Materials and Methods

Mouse experiments

Mice were maintained on a mixed 129S4/SvJae and C57Bl/6 background. The Pax7\(^{creER}\) allele (B6;129-Pax7\(^{creER}\)1(Eytm/SvJ)) has been described (16). R26\(^{fl/+}\); Zfp516\(^{fl/+}\) (B6;129 \(\times\) 1-Gt[ROSA]26Sor\(^{tm1(Enh)Wjt}\)) reporter mice (17) were obtained from the Jackson Laboratory. Kras\(^{LSL-G12D}\) (18) and Trp53\(^{fl/fl}\) mice (19) were obtained from Tyler Jacks and Anton Berns, respectively. The
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c-Metlox/lox mice (P78;129P2-MetimLSdf/J) were obtained from the Jackson Laboratory (20). All animal experiments were performed according to protocols approved by the Duke University (Durham, NC) and Carnegie Institution for Science (Baltimore, MD) Institutional Animal Care and Use Committee.

Sarcomas were generated in P7KP mice using i.p. injections of tamoxifen (Sigma-Aldrich) dissolved in ethanol and diluted in corn oil (10 μL of 20 mg/mL tamoxifen per g body weight). PBS, cardiotoxin (0.5 mg/mL dissolved in PBS), and HGF (2 μg/mL dissolved in PBS) were delivered intramuscularly (i.m.) at a volume of 25 μL. 5'-Ethynyl-2'-deoxyuridine (EdUrd) (EdUrd), a thymidine analog that serves as a marker of cell proliferation, was dissolved in PBS at a concentration of 0.5 mg/mL and administered as a 25×0.95% water objective using Leica image acquisition software. Images were processed using ImageJ software. Percentage of P-MET/YFP double positive cells was calculated for each muscle, then percentages were averaged and presented with SD. n = 3 muscles for 2 mice, over 1,000 cells counted for injured and uninjured muscles.

PCR

The details of the PCR protocol for detection of recombined and wild-type c-Met were kindly provided by Dr. Thorgerisson (National Cancer Institute, Bethesda, MD; ref. 20). Briefly, Quick-Load Taq 2X Master Mix (NEB) was used to assess c-Met recombination. The cycling parameters were 94°C for 30 seconds, 56°C for 30 seconds, and 68°C for 60 seconds for a total of 30 cycles. Primers Met-1 and Met-2 yield a Fioned (380 bp) and a WT (300 bp) band. Primers Met-1 and Met-3 yield a PCR product corresponding to the deleted allele (650 bp).

Primers

Met-1: 5'taaagaataatgggtcctcacc3'
Met-2: 5'ccaggtgggtccttaacctaatg3'
Met-3: 5'cagctctcaacagatgac3'

Statistical analysis

Survival curves were compared with the log-rank test using GraphPad Prism software. Statistical significance of values reported on bar graphs was determined using the Student t test.

Results

A temporally and spatially restricted model of STS

We previously developed a temporally and spatially restricted mouse model of STS using i.m. injection of an adenovirus expressing Cre recombinase (21), which has been useful for surgical and radiation therapy experiments (22, 23). However, adenovirus infects a heterogeneous population of cells. To generate a spatially restricted tumor model where the cell of origin is better defined, we delivered 4OHT by i.m. injection into the gastrocnemius muscle of the left hindlimb of P7KP mice (6) to try to restrict genetic recombination and subsequent sarcoma formation to the site of injection. We treated 14 P7KP mice with 1.0 μL of 50 μL of 10 mg/mL 4OHT dissolved in DMSO. As expected, all mice developed a tumor at the site of injection (Fig. 1A). However, we were surprised to find that the kinetics of tumor onset (median = 20 days, range 13–26 days) was faster than after i.p. tamoxifen (median 45 days, range 33–62 days; Fig. 2A; ref. 6). Evaluation of the tumors by histology revealed pleomorphic sarcomas characterized by spindle cells, epithelioid cells, or a mixture of both. The sarcomas were called pleomorphic rhabdomyosarcoma (RMS) if they stained positive for MyoD and myogenin (71% of tumors), and they were categorized as UPS if they lacked immunoreactivity for both differentiation markers (29% of tumors; Fig. 1A and B).

Intramuscular injection with DMSO injures muscle and accelerates STS formation

The kinetics of spatially restricted tumor formation in P7KP mice was significantly more rapid than that observed following systemic tamoxifen exposure, which suggested that i.m. injection of the DMSO vehicle accelerates sarcoma formation. To investigate this...
possibility, we treated P7KP mice with systemic tamoxifen along with concurrent i.m. injection of DMSO (vehicle for 4OHT; Fig. 2B). With this experimental approach, the recombination frequency following i.p. tamoxifen should be similar in Pax7-expressing cells throughout the mice. A cohort of mice was also treated with i.p. tamoxifen and concurrent i.m. saline to determine whether any effect observed with DMSO was specific to the vehicle or simply related to the i.m. injection itself.

Remarkably, 100% of P7KP mice treated with i.p. tamoxifen and i.m. DMSO developed a palpable tumor at the i.m. injection site in a median of 22 days (range 14–29 days; Fig. 2B), similar to the kinetics seen with 4OHT alone (Fig. 2A). In P7KP mice treated with i.p. tamoxifen and i.m. saline, 50% of mice developed an injection site sarcoma, but the time to tumor was significantly longer (median 41 days, range 40–48 days; Fig. 2B). These results reveal that DMSO accelerates sarcoma formation in P7KP mice. Of note, sarcomas developed at sites other than the injection with similar kinetics as P7KP mice treated with i.p. tamoxifen alone (data not shown).

To determine whether i.m. DMSO injection caused any change in the histologic appearance of muscle, littermates of the P7KP mice (P7P mice) were treated with i.m. DMSO and euthanized 1, 3, and 7 days following injection. I.m. saline was used as a negative control, and cardiotoxin, a component of cobra venom often utilized in muscle injury and regeneration studies (24), was used as a positive control. Evaluation of muscle treated with i.m. DMSO revealed significant cell death and neutrophil infiltration, consistent with injury to the skeletal muscle (Fig. 2C). As expected, massive cell death and inflammation were seen in cardiotoxin-treated muscle, and no significant changes were seen in saline-treated muscle (Fig. 2C).

Cardiotoxin-mediated injury also accelerates sarcoma formation in P7KP mice

The experiments described above indicate that i.m. injection of DMSO resulted in a tissue injury, leading to more rapid sarcoma formation. To further test this model, we next determined whether i.m. injection of cardiotoxin could promote sarcoma formation in P7KP mice following systemic tamoxifen because muscle injury following administration of cardiotoxin has been extensively characterized (Fig. 2C; ref. 24). Eight P7KP mice were treated with i.p. tamoxifen and concurrent i.m. cardiotoxin (Fig. 3B). Remarkably, all 8 mice developed palpable tumors at the cardiotoxin injection site with a median onset of 17 days (range 7–17 days). Thus, concurrent administration of systemic tamoxifen and injury with i.m. cardiotoxin in P7KP mice exerts temporospatial control over the formation of SIS at the injury site.

We next studied the temporal relationship between tamoxifen-mediated recombination of p53 and Kras and cardiotoxin-mediated tissue injury. A time-course experiment was performed whereby P7KP mice were injured with i.m. cardiotoxin at times before, after, and coincident with i.p. tamoxifen delivery (Fig. 3A).
and C). When cardiotoxin-mediated injury was performed either just before recombination with tamoxifen (up to 3 days before) or at any timepoint thereafter (up to 21 days after), an injection site sarcoma formed within 20 days of cardiotoxin administration with nearly 100% penetrance (Fig. 3B and D). No sarcomas formed following treatment with cardiotoxin alone, as seen in other studies (14). These results are consistent with a classic initiator/promoter model of sarcomagenesis whereby recombination with i.p. tamoxifen provides the initiating genetic insult, and injury serves as the promoter to drive sarcoma formation.

**The HGF/c-MET pathway promotes sarcoma formation in P7KP mice**

In the time course experiment, we were intrigued by the observation that recombined Pax7-expressing cells appeared to have reduced transformation potential until they were “activated” by cardiotoxin administration. Similar to recent experiments in squamous cell carcinoma (25) and intestinal cancer (26), we hypothesized that a key function of cardiotoxin might be to alter the activation state of Pax7-expressing satellite cells. We first assessed the number of proliferating satellite cells in P7KP mice following a single dose of systemic tamoxifen. For these experiments, P7KP mice (n = 3 per group) were also treated with i.m. saline or HGF, which has been shown to promote satellite cell proliferation both in vitro and in vivo (27, 28). Of note, the i.m. injection volume to the tibialis anterior (TA) muscle for these experiments was reduced to 25 μL in an effort to limit trauma related to the volume of injection (Supplementary Fig. S1A and S1B online). The mice were given two consecutive daily doses of EdUrd and euthanized on day 3 after the i.p. tamoxifen and the i.m. HGF/saline injections. We collected the TA muscles and stained 10 μm frozen sections for Pax7 and EdUrd (Supplementary Fig. S2A and S2B online). We counted 400 to 600 satellite cells per mouse and determined the percentage that was EdUrd⁺ (Pax7⁺).
Injury Promotes Sarcoma Formation

Figure 3. Tissue injury with cardiotoxin acts as a promoter to accelerate sarcoma formation following initiation by tamoxifen-mediated recombination. A, P7KP mice were treated with i.p. tamoxifen on day 0 followed by tissue injury with cardiotoxin injected into the gastrocnemius muscle on days 0, 7, and 21. B, injection site tumor-free survival is shown both post-tamoxifen and post-cardiotoxin. Of note, the curves overlap on the post-cardiotoxin survival curve, consistent with injury by cardiotoxin acting as a rate-limiting step for rapid sarcoma formation following recombination of Trp53fl/fl and KrasLSL-G12D in tamoxifen-treated mice. C, a similar experiment was performed where P7KP mice were treated with i.m. cardiotoxin 21, 7, 3, and 0 days before i.p. tamoxifen. D, injection site tumor-free survival is shown both post-cardiotoxin and post-tamoxifen. In contrast with when cardiotoxin is administered after tamoxifen, cardiotoxin administered >3 days before i.p. tamoxifen does not accelerate sarcoma formation, even when evaluated as the time-to-tumor post-tamoxifen. Taken together, these data support a model where tamoxifen-mediated recombination of Trp53fl/fl and KrasLSL-G12D acts as a classic initiator and tissue injury by cardiotoxin acts as a promoter to accelerate sarcoma formation; NS, not significant; ***, P < 0.001; ****, P < 0.0001.

EdUrd double positive) as a measure of satellite cell activation. Interestingly, the vast majority of Pax7+ cells were not proliferating in mice treated with i.m. saline despite deletion of p53 and activation of Kras in Pax7+ expressing cells throughout the animal (Fig. 4A). Administration of i.m. HGF caused a >3-fold increase in EdUrd uptake in satellite cells (Fig. 4A). Moreover, evaluation of specimens by H&E staining revealed no significant inflammatory infiltrate (Supplementary Fig. S2C online). Thus, HGF promotes proliferation of recombined satellite cells without the histologic hallmarks of tissue injury.

We next determined whether activation of recombined satellite cells by HGF is sufficient to promote tumor development at the site of injection after systemic tamoxifen. A total of 39 P7KP mice were treated with i.p. tamoxifen along with i.m. injection of 25 μL of either 40 μmol/L cardiotoxin (positive control), saline (negative control), or HGF (Fig. 4B). Of note, a concentration of 40 μmol/L cardiotoxin was used because the time-to-tumor was not statistically different than a concentration of 75 μmol/L (Supplementary Fig. S1C–S1E online). All cardiotoxin-treated mice developed a sarcoma at the injection site with a median onset of 15 days, whereas only 3 of 12 saline-treated mice developed a sarcoma at the injection site (Fig. 4C). In contrast, 11 of 13 mice treated with i.m. HGF developed a sarcoma at the injection site (Fig. 4C). Of note, the sarcomas arising in HGF-treated mice occur with a median onset of 37 days, which is slower than sarcomas that arise following treatment with cardiotoxin (Fig. 4D).

Next, we wished to determine whether the increased penetrance of sarcoma formation seen with HGF is mediated by the c-MET receptor rather than a nonspecific effect from the i.m. injection. We crossed P7KP mice to c-Metflox/flox mice (20) to cause c-Met deletion by CreER in satellite cells upon tamoxifen treatment. We treated 11 P7KP;c-Metflox/flox and 13 P7KP;c-Metflox/þ littermate control mice with systemic tamoxifen and i.m. HGF (Fig. 4E). We observed that P7KP mice with homozygous c-Met deletion no longer had an increased penetrance of tumor formation at the i.m. HGF injection site as compared with P7KP mice with only one copy of c-Met deleted (Fig. 4F and G). Of note, P7KP;c-Metflox/flox mice could not be followed beyond 2 to 3 months because they...
developed sarcomas at other sites. These data provide genetic evidence that HGF requires the c-MET receptor to increase the penetrance of sarcoma formation in P7KP mice.

c-MET has recently been shown to play an important role in satellite cell migration in the context of cardiotoxin-mediated injury (15), so we were interested to learn if it might also play a role in injury-induced sarcomas. We injected Pax7CreER<sup>fl</sup>/C3<sup>+/+</sup>; R26LSL-YFP<sup>YFP</sup>(P7Y) mice with i.m. cardiotoxin and assessed the levels of activated c-MET (P-MET) in satellite cells contained within injured and noninjured muscle (Fig. 5A). In P7Y mice injected with i.p. tamoxifen and i.m. cardiotoxin and euthanized 3 days after, the number of P-MET<sup>+</sup> cells within the YFP<sup>+</sup> population was 40-fold higher than that in uninjured muscle (Fig. 5B). This result emphasizes that c-MET is activated following acute muscle injury by cardiotoxin. Of note, the increased number of DAPI-stained nuclei seen in muscle injured by cardiotoxin (Fig. 5A) has been reported by others to be related to inflammation, fibroblast infiltration, as well as an increase in the number of activated satellite cells that are proliferating within the injury site (29–32). Indeed, IHC of muscle sections from injured P7Y mice contain numerous CD45<sup>+</sup> cells, consistent with a significant immune infiltrate (Fig. 5C). The presence of satellite cells (YFP<sup>+</sup> cells) lacking activated c-MET suggests that alternative pathways are likely to be involved in satellite cell activation in vivo (15).

We then tested whether c-MET signaling might be a critical mediator of the sarcoma-promoting effect of cardiotoxin. A total of 20 mice (10 P7KP;c-Met<sup>fl/fl</sup> and 10 P7KP;c-Met<sup>lox/lox</sup>) were treated with systemic tamoxifen along with i.m. cardiotoxin (Fig. 6A). As expected, all of the P7KP;c-Met<sup>fl/fl</sup> mice developed sarcomas at the i.m. injection site with rapid kinetics (Fig. 6B). P7KP;c-Met<sup>lox/lox</sup> mice also developed a sarcoma at the site of i.m. cardiotoxin administration, but the kinetics were much slower and similar to those observed in spontaneously developing tumors after i.p. tamoxifen (Fig. 6B, median = 42 days). This result reveals that...
c-MET signaling is required for cardiotoxin-mediated promotion of sarcoma formation in P7KP mice. Once formed, sarcomas grew at similar rates regardless of whether or not c-MET was present (Fig. 6C). Notably, sarcomas arising in P7KP; c-Met<sup>fl<sup>ox/</sup>fl<sup>ox</sup></sup> mice had full recombination at both c-Met loci, confirming that sarcomas arise despite the deletion of c-Met (Fig. 6D).

As a corollary experiment, we treated 6 P7KP; c-Met<sup>fl<sup>ox/</sup>fl<sup>ox</sup></sup> and 10 P7KP; c-Met<sup>fl<sup>ox</sup>/+</sup> controls with i.m. 4OHT and again observed that the median time to tumor at the site of injection was significantly faster in those mice that retained a copy of wild-type c-Met (Supplementary Fig. S3 online). This further proves that the c-MET pathway is required for sarcoma promotion in the context of injury in P7KP mice.

**Discussion**

The P7KP mouse model of STS is a unique system to study the role of injury in tumorigenesis because it provides tight control of the timing of both genetic mutation (i.p. injection of tamoxifen) and injury (i.m. injection of cardiotoxin) before tumor formation. Other injury-mediated sarcoma model systems have been described, but they all lack the ability to control the timing of oncogenic mutations in a genetically engineered organism. Perhaps the most noteworthy model system was described by the laboratory of Dr. Bissell (Lawrence Berkeley National Laboratory, Berkeley, CA; refs. 8–10). They described a series of experiments in chickens infected with Rous sarcoma virus. The chickens developed sarcomas at the site of virus injection in the wing despite systemically elevated viral titers. However, the chickens developed sarcomas in the contralateral wing after wounding with a clip or suture material. Similar findings were seen in transgenic mice constitutively overexpressing the v-jun oncogene (11–13). The transgenic mice did not have any demonstrable phenotype with the exception of sarcoma formation at the site of ear tagging and tail clipping. More recent examples include the observation that recurrent muscle injury with cardiotoxin gives rise to STS at the site of injury in p53-null mice (14). Attempts to generate urothelial tumors in a mouse model containing inducible activation of Kras and loss of p53 resulted in STS at the suture site, though this was reported as an incidental finding as the intent was to generate a genitourinary tumor (33). Finally, neurofibromas were found to preferentially develop at the site of nerve injury in a mouse model of neurofibromatosis type 1 (34).

The rapid kinetics of tumor formation in P7KP mice treated with systemic tamoxifen and i.m. cardiotoxin demonstrates that injury acts as a classic promoter of STS in a mammalian system.
Most Pax7+ cells remain quiescent despite loss of p53 and expression of oncogenic Kras (Fig. 4A), but cardiotoxin breaks their quiescence and promotes tumor formation in a process dependent on c-MET signaling. HGF is also able to break quiescence of satellite cells harboring p53 mutations and promote sarcoma formation (Fig. 4A–D and Supplementary Fig. S2 online), but the level of HGF used in our experiment was not sufficient to speed up the kinetics. It is also possible that the expression of other growth factors in addition to HGF that signal through c-MET is required to promote sarcoma formation. For example, it has been widely reported that the c-MET pathway has a role in sarcoma formation. Taulli and colleagues showed that cell lines derived from human embryonal RMS and alveolar RMS expressed high levels of c-MET and HGF (40). Other groups have shown that the HGF/c-MET pathway is upregulated in osteosarcoma, chondrosarcoma, and leiomyosarcoma (41, 42), and elevated HGF/c-MET expression in human synovial sarcoma has been correlated with poor prognosis in patients (43).

In addition, HGF/c-MET signaling has been reported to play a role in sarcoma formation. In Ink4a/Arf knockout mice over-expressing HGF under the direction of the metallothionein promoter develop embryonal RMSs with a mean onset of 3.3 months. In this study, the skeletal muscle of 6- to 10-week-old mice had hyperplastic satellite cells, consistent with a role for HGF in tumor initiation by promoting proliferation of cells lacking Ink4a/Arf (39). HGF/c-MET signaling has also been implicated in many different types of human sarcoma. Taulli and colleagues showed that cell lines derived from human embryonal RMS and alveolar RMS expressed high levels of c-MET and HGF (40). Other groups have shown that the HGF/c-MET pathway is upregulated in osteosarcoma, chondrosarcoma, and leiomyosarcoma (41, 42), and elevated HGF/c-MET expression in human synovial sarcoma has been correlated with poor prognosis in patients (43).

In P7KP mice, we propose that HGF/c-MET signaling is acting as a classic promoter, activating satellite cells in the context of cardiotoxin injury. Our finding that 21 days can pass between recombination of p53 and Kras and injury by...
cardiotonic suggests that satellite cells can harbor oncogenic mutations without progression to sarcoma formation. The rarity of sarcomas in humans might be partially explained by the requirement for this additional "activation" step, whereby quiescence prevents cells that harbor oncogenic mutations from forming sarcomas.

Our data also lend credence to patient reports of a localized injury causing their sarcoma (44). In his landmark 1919 textbook, Neoplastic Diseases, James Ewing wrote that "sarcoma commonly develops after a single blow." When patients report a history of trauma before the development of sarcoma at the same location, such claims are difficult to substantiate in a rigorous manner and are frequently ignored by physicians. Here, we report on the development of a temporally and spatially restricted mouse model of STS that allows for the precise timing of genetic mutations and tissue injury. Our data demonstrate that injury promotes sarcoma formation in a process dependent on HGF/c-MET signaling. Although "a single blow" is not likely to cause sarcoma, our results indicate that injury might promote sarcoma development by activating quiescent progenitor cells that have acquired oncogenic mutations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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