Brief Communications

Novel Animal Models of Acute and Chronic Cancer Pain: A Pivotal Role for PAR2

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Targeted therapy to prevent the progression from acute to chronic pain in cancer patients remains elusive. We developed three novel cancer models in mice that together recapitulate the anatomical, temporal, and functional characteristics of acute and chronic head and neck cancer pain in humans. Using pharmacologic and genetic approaches in these novel cancer models, we identified the interaction between protease-activated receptor 2 (PAR2) and serine proteases to be of central importance. We show that serine proteases such as trypsin induce acute cancer pain in a PAR2-dependent manner. Chronic cancer pain is associated with elevated serine proteases in the cancer microenvironment and PAR2 upregulation in peripheral nerves. Serine protease inhibition greatly reduces the severity of persistent cancer pain in wild-type mice, but most strikingly, the development of chronic cancer pain is prevented in PAR2-deficient mice. Our results demonstrate a direct role for PAR2 in acute cancer pain and suggest that PAR2 upregulation may favor the development and maintenance of chronic cancer pain. Targeting the PAR2–serine protease interaction is a promising approach to the treatment of acute cancer pain and prevention of chronic cancer pain.

Introduction

Cancer patients suffer from acute and chronic pain, with components of both persistent and breakthrough pain (Portenoy and Lesage, 1999). Head and neck cancer patients experience pain early in the disease process and orofacial pain is often the presenting symptom for oral squamous cell carcinoma (SCC) patients (Marshall and Mahanna, 1997). The onset of orofacial pain that is exacerbated during function has been associated with the transition of oral precancer to cancer (Lam and Schmidt, 2011). Debilitation of oral function, secondary to cancer pain, worsens with disease progression in head and neck cancer patients (Connelly and Schmidt, 2004). Animal models suggest a role for calcitonin gene-related peptide (CGRP), substance P, ATP (P2X3), vanilloid (TRPV1), endothelin (ET-1), and serine protease (PAR2) receptor mechanisms in head and neck cancer pain (Viet and Schmidt, 2012). However, these existing animal models in ectopic tissue sites, such as the mouse hindpaw, using reflex responses as dependent pain measures fail to reflect the anatomical, temporal and functional features of acute and chronic head and neck cancer pain. Therefore, we developed three novel anatomically appropriate head and neck cancer-specific models in mice, which together parallel the temporal progression and functional impairment experienced by humans with acute and chronic head and neck cancer.

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Our novel head and neck cancer-specific models have the potential to elucidate the mechanisms for the transition from acute to chronic cancer pain. Since we recently identified a novel role for PAR2 in mediating acute cancer pain in an ectopic hindpaw model (Lam and Schmidt, 2010), we tested the hypothesis that PAR2 similarly mediates acute cancer pain in our head and neck cancer-specific model, and hypothesized that chronic head and neck cancer pain is associated with PAR2 upregulation in peripheral nerves. Here we show evidence for a crucial PAR2-dependent mechanism in both acute and chronic head and neck cancer pain.

Materials and Methods

Cell culture

Human oral SCC (HSC-3, ATCC) and normal oral keratinocyte (NoK) cell lines were prepared as previously described (Lam and Schmidt, 2010). Briefly, the cell lines were grown independently to confluence, the media was replaced with Defined Keratinocyte–Serum Free Media, and further incubated for 72 h before use in the models described below.

Head and neck cancer nociceptive models

PAR2-deficient (PAR2 $^{-/-}$) mice (6–8 weeks old), originally from Jackson Laboratory (strain: B6.Cg-*F2rl1*^{tm1Mslb}/J;), were established on a C57BL/6 background together with their wild-type littermates at the University of California, San Francisco (UCSF) Animal Care facility as previously described (Lam and Schmidt, 2010). All experiments, except where indicated, were performed on adult female PAR2 $^{-/-}$ mice and their age- and sex-matched wild-type littermates. Mice were housed in a temperature-controlled room on a 12 h light cycle, with unrestricted access to food and water. All procedures adhered to the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Zimmermann, 1983) guidelines and were approved by the UCSF Committee on Animal Research.

Cancer-induced functional impairment (mechanical allodynia) was studied using the Dolognawmeter, a validated operant assay for quantifying cancer pain-induced gnawing dysfunction as well as its reversal

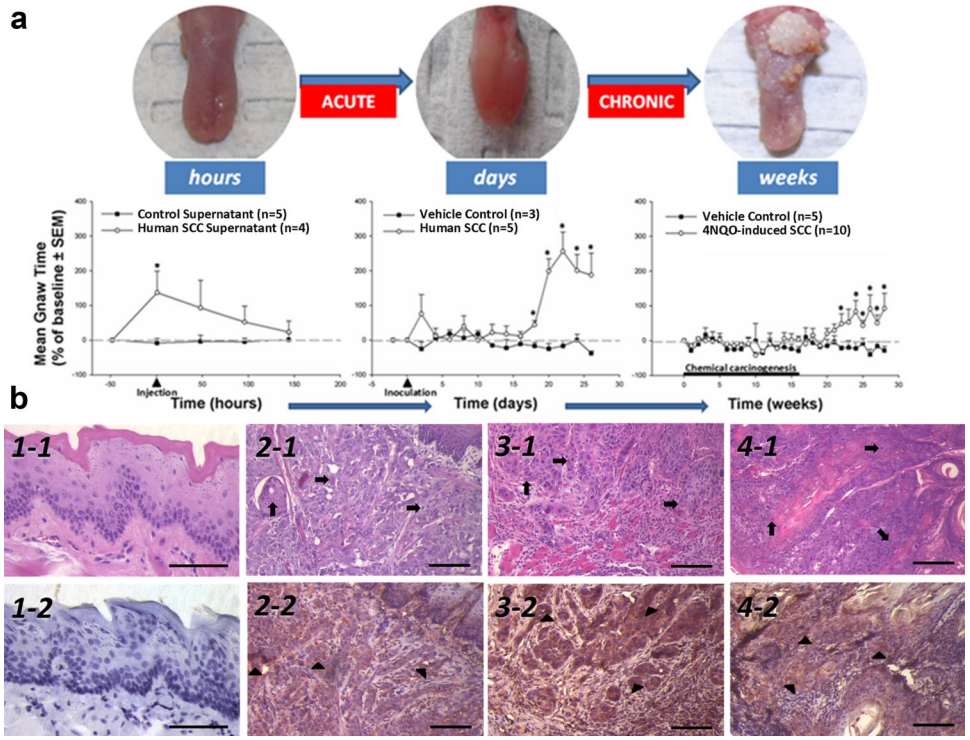

Figure 1. Three novel head and neck cancer models together reflect the anatomical, temporal and functional characteristics of head and neck cancer pain. ***a***, Acute pain (left), lasting days, was induced by injecting supernatant from human cancer (SCC) into the tongue; persistent pain (middle), lasting weeks, was induced by human SCC xenograft inoculation into the tongue; and chronic pain from 4NQO-chemical carcinogenesis (right), persisted for months. ***b***, Trypsin immunoreactivity is increased in the cancer microenvironment as shown by representative hematoxylin and eosin (top), and corresponding trypsin immunoreactivity (bottom) images. Normal tongue mucosa (***b1-1***) has no/minimal trypsin immunoreactivity (***b1-2***), whereas SCC cells (arrows) from human cancer xenografts (***b2-1***) and 4NQO (***b3-1***) in wild-type mice show intense trypsin immunoreactivity (arrowheads) (***b2-2*** and ***b3-2***, respectively). 4NQO-induced cancers (***b4-1***) in PAR2 $^{-/-}$ mice also show intense trypsin immunoreactivity (arrowheads, ***b4-2***). *$p < 0.05$, RM ANOVA. Scale bars, 100 μm.

with opioid analgesics, as previously described (Dolan et al., 2010). Briefly, all behavioral testing was performed at ~7:00 PM and mice underwent 10 training trials. After the first few trials, each mouse reached consistency for the amount of gnaw time required to sever two dowels, with the mean of the final three training trials used as a baseline. Following the determination of baseline gnaw times the three cancer pain models were produced.

Acute cancer pain. A novel acute oral cancer pain model was developed by injecting oral SCC supernatant into an orthotopic oral tissue site. Functional allodynia was compared between wild-type mice receiving injections of supernatant from SCC ($n = 4$) or NoK ($n = 5$). Fifty microliters of SCC or NoK supernatant was injected over a 5 s period, into the left lateral tongue under isoflurane (Summit Medical Equipment Company) general anesthesia. Dolognawmeter measurements were recorded in awake mice at 45 min postinjection of supernatant, and every 48 h thereafter. We then investigated whether the mechanism of acute cancer-induced pain is PAR2 dependent, by comparing nociceptive responses of wild-type and PAR2 $^{-/-}$ littermate mice ($n = 4$/group), following injection of SCC supernatant.

To assess whether trypsin alone is sufficient to induce the functional allodynia observed in the acute pain model, wild-type mice ($n = 5$/group) received 50 μl injections with a single dose of trypsin (10, 100, 300, or 600 μg) or saline control, into the left lateral tongue, and Dolognawmeter measurements were taken. To determine whether this trypsin-mediated mechanism was PAR2 dependent, mice received a single 50 μl injection of the largest trypsin dose (600 μg) and the nociceptive responses were compared between wild-type and PAR2 $^{-/-}$ littermate mice ($n = 5$/group).

Persistent cancer pain. A novel persistent oral cancer pain model was developed by injecting human oral SCC cells into an orthotopic oral tissue site of adult female BALB/c nude mice (6–8 weeks old, Charles River Laboratories). Fifty microliters of 3×10^5 SCC cells suspended in vehicle (1:2 mixture of DMEM and Matrigel; Becton Dickinson), or vehicle alone, was injected into the left lateral tongue under isoflurane anesthesia. Dolognawmeter measurements were made in awake mice every 48 h after inoculation.

To elucidate the effect of trypsin on the mechanism of cancer-induced persistent pain, mice received 20 μl intratumor injections of soybean trypsin inhibitor (SBTI, 1 μg/μl; $n = 5$, Sigma), or saline vehicle, and nociceptive responses were compared.

Chronic cancer pain. To establish SCC in mice that enabled examination of chronic pain induced by head and neck cancer, mice ingested the carcinogen 4-nitroquinoline 1-oxide (4NQO; 100 μg/ml; Sigma) with their drinking water on an unrestricted basis for 16 weeks. Water was changed weekly with freshly prepared 4NQO dissolved in propylene glycol (5 mg/ml) (Tang et al., 2004). Control mice received water containing the equivalent dilution of propylene glycol alone. At the end of this period, functional allodynia, as a result of progressive 4NQO-induced carcinogenesis, was assessed using the Dolognawmeter once a week, for 12 weeks.

Serine proteases in cancer microenvironment

Trypsin mRNA. We used RT qPCR to quantify and compare trypsin transcript expression levels in normal (NoK) and cancer (HSC3) cells used in the acute and persistent pain models. Total RNA isolation and reverse transcription was performed with DNA/RNA (Qiagen Inc.) and High Capacity cDNA Reverse Transcription (Applied Biosystems Inc.) Kits according to the manufacturer's protocol. All qPCR assays were performed in triplicate with a TaqMan Gene Expression Assay Kit (Applied Biosystems Inc.). Cycling conditions consisted of one cycle of 50°C for 2 min followed by 95°C for 10 min, and then 40 cycles of 95°C for 15 s followed by 60°C for 1 min. The housekeeping gene β-glucuronidase served as the internal control. Relative quantification analysis of gene expression data was conducted according to the $2-^{\Delta\Delta CT}$ method.

Trypsin immunopositivity. Trypsin immunohistochemistry was performed on 8 μm sections of formalin-fixed, paraffin-embedded tissue

specimens deparaffinized by standard immunohistochemical techniques. Microwave antigen unmasking was performed using Antigen Retrieval Solution (Dako). Sections were incubated with the primary rabbit polyclonal anti-trypsin rabbit antibody [0.4 μg/ml, H-101: sc-67387 (raised against amino acids 39–140 mapping near the N terminus of Trypsin-1 of human origin) for persistent model; and M-60: sc-67388 (raised against amino acids 1–60 mapping at the N terminus of Trypsin-1 of mouse origin) for chronic model; Santa Cruz Biotechnology] at room temperature for 2 h. Immunoreactions were visualized with NovaRED substrate kit (Vector Laboratories), and sections counterstained with Hematoxylin QS. Trypsin immunoreactivity was abolished when primary antibody was preincubated with 20-fold excess immunogenic peptide for 24 h at 4°C before staining commenced. Omission of the primary antibody also failed to demonstrate trypsin immunoreactivity.

PAR2 upregulation in cancer pain

Trigeminal ganglia (TGs) were dissected aseptically from mice in the acute, persistent and chronic cancer pain models at 1, 4, and 25 weeks, respectively. Ganglia were washed with cold (4°C) modified Hanks' balanced salt solution, snap frozen in liquid nitrogen, and stored at −80°C until further processing.

PAR2 immunopositivity. The primary rabbit polyclonal anti-PAR2 antibody (0.4 μg/ml H-99: sc-5597, Santa Cruz Biotechnology) was raised against amino acids 230–328 of PAR2 of human origin and stains a single 80 kDa band on Western blot (manufacturer's technical information). Following primary antibody incubation for 2 h, TGs were incubated with secondary anti-rabbit FITC-conjugated antibody (7.5 μg/ml, Jackson ImmunoResearch Laboratories Inc.) for 1 h, at room temperature. Coverslips were mounted on slides in Gel-Mount (Biomeda Corp.) and visualized on a Nikon Eclipse E600 microscope using epifluorescence. PAR2 immunoreactivity in normal pain-free mice was compared with that in cancer mice showing signs of cancer pain, with CellProfiler (Carpenter et al., 2006) by a blinded observer. Image intensity was rescaled from 0 to 1 by dividing all image pixels by the maximum possible intensity value. PAR2 immunofluorescence intensity was quantified in mean intensity units (average pixel intensity within a cell). Only neurons that did not overlap with other cells and had a visible nucleus were used for image analysis. Controls for PAR2 immunoreactivity included replacement of primary antibody with similarly diluted normal rabbit serum and omission of primary antibody. As an additional negative control, incubation of the primary antibody with TGs from PAR2$^{-/-}$ littermate mice failed to demonstrate PAR2 immunoreactivity.

PAR2 mRNA. To further implicate PAR2 upregulation in the progression of cancer pain, we used RT qPCR to quantify and compare PAR2 transcript expression levels in the TGs from mice with and without cancer pain in the three pain models. The same methodology was used as above for the trypsin mRNA assay.

Statistical analysis

Data are reported as mean ± SE. Paired *t* test and repeated-measures (RM) ANOVA (Bonferroni's or Dunn's) were used as appropriate ($p < 0.05$ considered to reflect statistical significance).

Results

Cancer models in animals together recapitulate anatomical, temporal, and functional characteristics of acute and chronic cancer pain in humans

We developed three novel head and neck cancer models that exploit impaired oral function caused by cancer pain, to demonstrate cancer-induced mechanical allodynia. In the acute pain model (Fig. 1*a*, left), mice exposed to supernatant from human SCC experience pain that lasts for hours to days. Persistent pain, which lasts weeks, is modeled by response to human SCC xenograft inoculation (Fig. 1*a*, middle). In the model for chronic pain (Fig. 1*a*, right), SCC, produced from 4NQO carcinogenesis, induces cancer pain that persists beyond weeks to months.

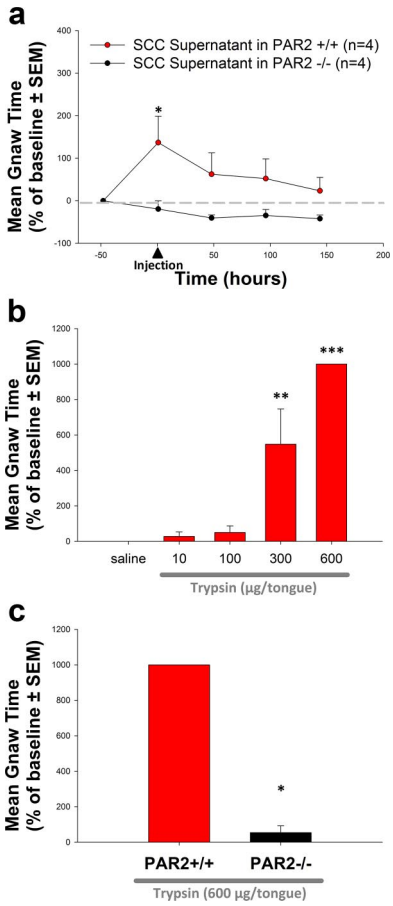

Figure 2. Elevated trypsin levels contribute to cancer-induced acute functional allodynia in a PAR2-dependent manner. ***a***, Human cancer (SCC) supernatant-induced acute functional allodynia was absent in PAR2$^{-/-}$ mice. ***b***, ***c***, Peripheral application of trypsin induced similar acute allodynia in a dose-dependent manner in wild-type mice (***b***) but was absent in PAR2$^{-/-}$ mice (***c***). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, RM ANOVA.

Trypsin levels increased in cancer microenvironment

We previously showed head and neck cancer cells release serine proteases, which contribute to acute cancer pain (Lam and Schmidt, 2010). To evaluate the role for the serine protease trypsin in more persistent and chronic cancer pain, we examined trypsin immunoreactivity in tongue cancers known to induce persistent (Fig. 1*b2*) and chronic (Fig. 1*b3*) pain. Head and neck cancer cells produce and secrete trypsin in abundance, as evidenced by the intense cytoplasmic and diffuse extracellular trypsin immunoreactivity in the tongue cancers.

Increased trypsin levels in cancer microenvironment contribute to acute cancer pain in a PAR2-dependent manner

Supernatant from human SCC, containing high levels of serine proteases such as trypsin (Lam and Schmidt, 2010), induced acute functional allodynia when injected into the tongue of wild-type mice (Fig. 1*a*, left). Functional allodynia, as indicated by increased gnaw time, began at 45 min postinjection of SCC supernatant and lasted ~24 h. In contrast, supernatant from control NoK had no effect.

Peripheral application of trypsin into the tongue induced similar acute functional allodynia in a dose-dependent manner in wild-type mice (Fig. 2*b*) suggesting that trypsin alone secreted by cancer cells is sufficient to induce acute cancer-related pain. Functional allodynia induced by SCC supernatant (Fig. 2*a*) and

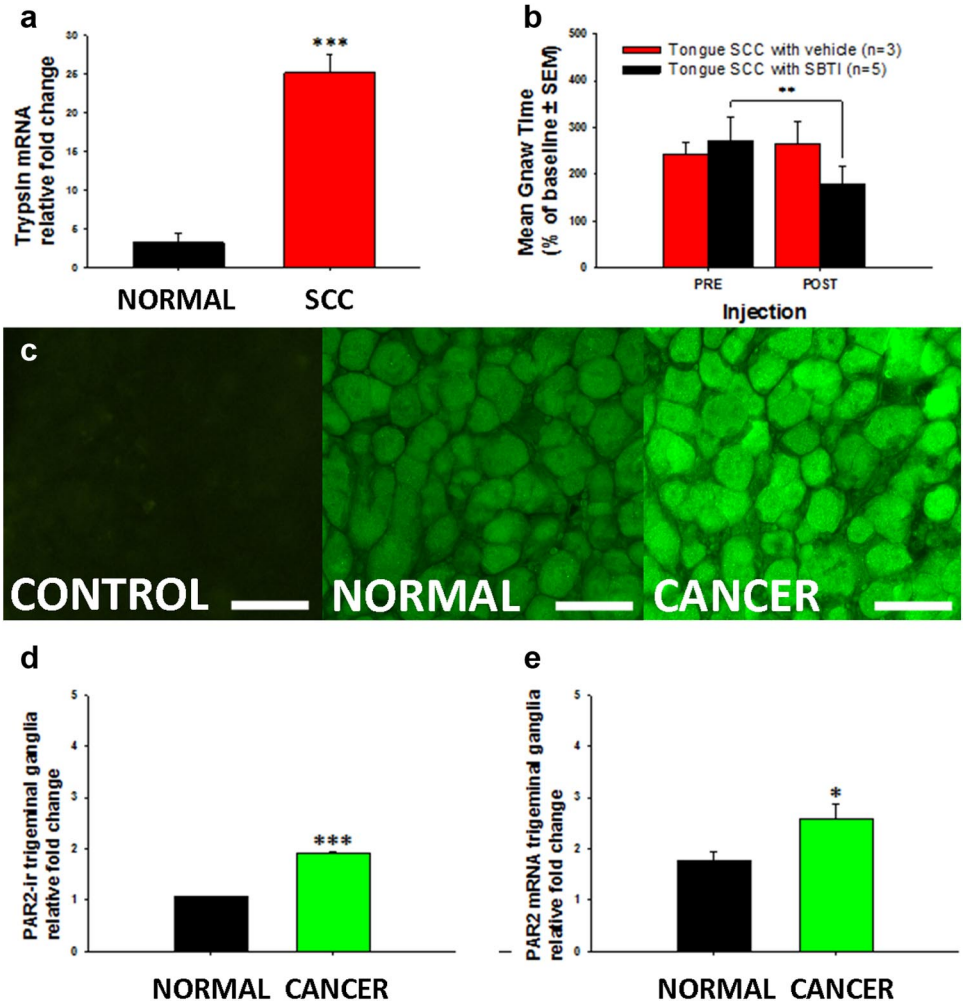

Figure 3. Elevated trypsin levels contribute to cancer-induced persistent functional allodynia and are associated with PAR2 upregulation in TGs. ***a***, Trypsin mRNA levels were markedly upregulated in cancer cells (HSC3) relative to normal control (NoK). ***b***, Persistent functional allodynia induced by human cancer cell (SCC) inoculation is attenuated by local injection of trypsin inhibitor (SBTI) (**$p < 0.05$, paired *t* test). ***c***, Representative PAR2 immunofluorescence images in TGs of omission control (left), normal (middle) and cancer (right) mice. ***d***, ***e***, PAR2 immunoreactivity (***d***) and PAR2 (***e***) mRNA levels were markedly upregulated in the TGs of cancer mice relative to normal mice. *$p < 0.05$, ***$p < 0.001$, RM ANOVA. Scale bars, 100 μm.

trypsin (Fig. 2*c*) was absent in PAR2$^{-/-}$ mice, supporting a PAR2-dependent mechanism of acute cancer pain. There were no differences in baseline gnaw times for mice in any of the treatment and control groups ($p > 0.05$, RM ANOVA).

Increased trypsin levels in cancer microenvironment contribute to persistent cancer-induced pain

We produced head and neck cancer in mice by inoculating human SCC cells into the tongue; these mice subsequently develop cancer and associated pain over days to weeks (Fig. 1*a*, middle). The human SCC used in this model show markedly elevated trypsin mRNA levels compared with normal control cells (Fig. 3*a*). Functional allodynia began at day 18 post-inoculation of SCC and persisted for 8 d (Fig. 1*a*, middle). Injection of SBTI into the tongue tumor attenuated this persistent pain (Fig. 3*b*), further reinforcing the nociceptive role of serine proteases, trypsin in particular, in the cancer microenvironment. There were no differences in baseline gnaw times for mice in any of the treatment and control groups ($p > 0.05$, RM ANOVA).

Chronic cancer-induced pain is PAR2 dependent

Chronic functional allodynia that lasted months, and worsened with disease progression, was induced in wild-type mice with head and neck cancer (Figs. 1*a*, right; and 4*a*). Functional allodynia began at 4–6 weeks post-4NQO chemical carcinogenesis treatment and continued to worsen for the remaining 2 months. In contrast, cancer-induced allodynia was completely absent in PAR2$^{-/-}$ mice, despite progression of SCC (Fig. 4*a*). There were no differences in baseline gnaw times for mice in any of the treatment and control groups ($p > 0.05$, RM ANOVA).

There was no difference in PAR2 immunoreactivity nor PAR2 mRNA expression in the TGs of mice following SCC supernatant or control NoK supernatant in the acute pain model ($p > 0.05$, RM ANOVA). In contrast, both PAR2 immunoreactivity and mRNA expression were significantly upregulated in the TGs of cancer-bearing mice, compared with normal cancer-free mice, in the persistent pain model (Fig. 3*c–e*). PAR2 immunoreactivity and mRNA expression were even more markedly upregulated in the TGs of cancer-bearing mice in the chronic pain model (Fig. 4*c–e*).

Discussion

We developed three novel head and neck cancer models in mice that together recapitulate the anatomical, temporal and functional characteristics of acute and chronic cancer pain in humans. The combination of these models allowed us to study the role of

candidate nociceptive mediators, and receptor mechanisms involved in the development and progression of head and neck cancer pain. The novel orthotopic injection of cancer supernatant alone in the acute cancer pain model allowed the nociceptive effect of extracellular products to be distinguished from the effects of cellular invasion, tumor mass, and systemic illness that accompany carcinogenesis. The persistent cancer pain model simulates the rapidly growing and destructive cancers that are often extremely painful in humans, as mice develop head and neck cancer and associated pain following inoculation of human cancer cells in the tongue. However, the 4NQO-induced carcinogenesis model best mimics the natural disease progress of head and neck cancer pain in humans. Carcinogens in tobacco induce carcinogenesis through the formation of DNA adducts and 4NQO is a water-soluble quinoline derivative that causes DNA adduct formation (Tang et al., 2004). Long-term treatment with 4NQO produces preneoplastic and neoplastic lesions in mice that parallel the development of head and neck cancer in humans. These sequential changes in epithelial cells exhibit similar histological, molecular, and chromosomal alterations as observed in human carcinogenesis, making 4NQO-induced carcinogenesis an ideal chronic head and neck cancer progression model (Tang et al., 2004). We now show that it is also a suitable model to study the development of chronic cancer pain that persists for months. Since head and neck cancer-induced orofacial pain, that is exacerbated during function, develops early in carcinogenesis and worsens with disease progression, our validated operant assay measuring pain-induced functional impairment may better reflect clinical cancer pain than previous animal models reliant on innate reflexes as dependent measures of cancer pain.

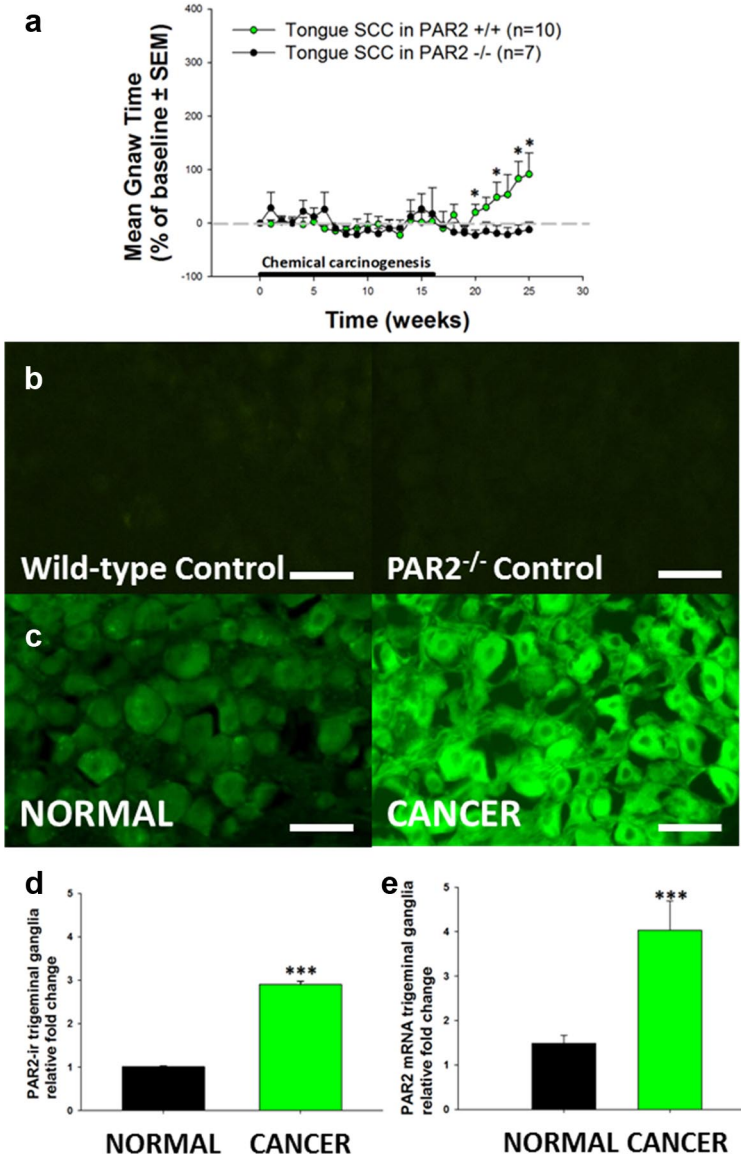

Figure 4. Cancer-induced chronic functional allodynia is PAR2 dependent. ***a***, Wild-type mice developed head and neck cancer (SCC) following 4NQO-chemical carcinogen exposure, engendering chronic functional allodynia. In contrast, chronic allodynia was absent in PAR2 $^{-/-}$ mice, despite their progression to SCC. ***b***, Representative PAR2 immunofluorescence images in TGs of wild-type (omission control) (left) and PAR2 $^{-/-}$ (negative control) (right) mice. ***c***, Representative PAR2 immunofluorescence images in TGs of normal (left) and cancer (right) mice. ***d***, ***e***, PAR2 immunoreactivity and (***e***) PAR2 mRNA levels were markedly upregulated in the TGs of cancer mice relative to normal mice. $*p < 0.05$, $***p < 0.001$, RM ANOVA. Scale bar, 100 μm.

Proteolytic activity is critical to carcinogenesis, and the cancer microenvironment is replete with various proteases. Cancer-associated serine proteases, such as trypsin, released during the early stages of tissue invasion may directly activate PAR2 on nociceptive afferents and result in acute pain that is spontaneous and exacerbated during function. PAR2 activation may also sensitize other nociceptive receptors such as TRPV1 and TRPV4 (Dai et al., 2004; Grant et al., 2007). With continued cancer cell proliferation and mediator release, nociceptive afferents may be persistently activated or sensitized and consequently maintain a persistent pain state. PAR2 is upregulated following various inflammatory and ischemic insults (Nystedt et al., 1996; Striggow et al., 2001). Mediators such as interleukin-1β, tumor necrosis factor-α, and trypsin can upregulate PAR2 (Ritchie et al., 2007). PAR2 upregulation in dorsal root ganglia has been associated with thermal hyperalgesia following chronic pancreatitis (Zhang et al., 2011) and cAMP-dependent neuronal hyperexcitability following chronic nerve compression (Huang et al., 2012). Cancer-induced PAR2 upregulation in TGs may similarly alter pain processing and mediate the progression to chronic cancer pain. The complete absence of cancer-induced functional allodynia in mice lacking PAR2 clearly shows the critical involvement of PAR2 in acute and chronic cancer pain.

PAR2 is emerging as a therapeutic target for several diseases including arthritis, asthma, colitis, neurodegenerative conditions, tumor invasion and cardiovascular diseases (Ramachandran et al., 2012). Our findings now suggest targeting PAR2 and specific serine proteases in the cancer microenvironment may be a promising approach to treating acute cancer pain and may prevent the progression to chronic cancer pain. The degree of trypsin inhibitor-induced cancer pain attenuation in the present

study may have particular clinical relevance since trypsin inhibition has similar efficacy to that previously demonstrated with systemic morphine (Dolan et al., 2010). Various approaches for abrogating PAR2 signaling include: blocking docking of the tethered ligand sequence with a small-molecule receptor antagonist; occluding the tethered ligand cleavage site with a PAR2 antibody; or interfering with interactions between PAR2 and its cognate intracellular signal transducer proteins using cell-penetrating peptides (Ramachandran et al., 2012). Other therapeutic strategies may include inhibition of PAR2-activating serine proteases to prevent the generation of the tethered ligand (Hansen et al., 2005), or siRNA targeting (Ferrell et al., 2003) to downregulate PAR2 expression.

To date, the following PAR2 antagonists have been validated in various animal models but have not yet progressed to clinical trials: GB88, ENMD1068, AC-264613, AC-55541 and K14585 (Ramachandran et al., 2012). Targeting PAR2 has not been straightforward given its broad involvement in normal and pathological tissue function. PAR2 is expressed predominantly in small- and medium-sized dorsal root and TG neurons (Zhu et al., 2005; Denadai-Souza et al., 2010); however, it is also expressed in CNS neurons and astrocytes in various regions of the brain (Striggow et al., 2001; Wang et al., 2002). PAR2 activation in the brain is linked to neuronal excitability, synaptic transmission and plasticity (Lohman et al., 2009; Gan et al., 2011), suggesting PAR2 signaling may exert both neuroprotective and neurodegenerative effects. Similarly, although the majority of data suggest PAR2 activation has a proinflammatory role in the respiratory and gastrointestinal tracts, there are also data supporting bronchorelaxant and anti-inflammatory roles (Cocks et al., 1999; Moffatt et al., 2002). Thus, in considering the therapeutic use of PAR2 antagonists in disease states, such as cancer pain, it will be important to balance its potential beneficial and adverse pharmacologic effects.

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