Supplementary methods

Primary human Schwann cells and cell lines

Human Schwann Cells (hSC) were purchased from ScienCell (Carlsbad, CA, USA) and are derived from human sciatic nerves as primary cells and can be cultured up to a maximum of 10 passages. hSC were cultivated according to the instructions of the manufacturer. For collection of PCa cell supernatants, PCa cells (PCC) were allowed to reach 80% confluence in their routine complete growth medium, and the medium was replaced with serum-free cancer cell medium and collected after 48 hours. hSC were cultivated in hSC medium with 5% fetal calf serum (FCS), 1% penicillin/streptomycin and 1% SC growth supplements (all from ScienCell, Carlsbad, CA, USA) in poly-D-lysine hydrobromide (40 mg/m^2, Sigma Aldrich, Taufkirchen, Germany) coated plates or 13mm coverslips. The protein concentration of all whole cell lysates and supernatants was measured with the BCA protein assay (Pierce Chemical Co., Rockford, IL, USA. The human pancreatic cancer cell lines Capan1, Colo357 and SU86.86, the human colon cancer (CCa) cell lines HCT-116 and HT29, and the human T-lymphocyte cell line Jurkat were purchased from ATCC (Rockville, MD, USA). The human glioblastoma cell line LN229 was a kind gift by Prof. J. Schlegl (Department of Neuropathology, TU München, Munich, Germany). T3M4 PCa cell line was a kind gift by Dr. R. S. Metzgar (Durham, NC, USA). The cell lines were routinely grown in complete medium, i.e. DMEM for LN229 and HT29, McCoy's 5A medium for HCT-116, and RPMI-1640 medium for the remaining cell lines (Gibco, Invitrogen, Karlsruhe, Germany).

Patients, tissues and pain severity classification

Among the pancreatic ductal adenocarcinoma patients (n=30, male/female = 20/10, median age = 63.5 yrs), there were 7 patients with stage IIa, 22 patients with stage IIb and 1 patient with stage III pancreatic cancer according to the current international classification of the

UICC. Tumour grading was “well differentiated” in 2 cases, “moderately differentiated” in 15 cases and “poorly differentiated” in 13 cases. The resected pancreatic tissue samples were divided into parts which were immediately fixed in 4% paraformaldehyde followed by paraffin-embedding, as described previously.[1] The pain score (pain intensity and frequency) was prospectively registered prior to the operation. Pain intensity was scored by using a short scale: 0 = none, 1= mild, 2 = moderate and 3 = strong pain. Pain frequency was scored as 3 = daily, 2 = weekly and 1 = monthly. To calculate the severity of pain, pain intensity and pain frequency of each individual were multiplied. According to the final pain score, the patients were divided into three groups: Pain I (0) representing the group of patients without pain, Pain II (1-3) patients who suffered from mild pain and Pain III (4-9), with moderate to severe pain.[2, 3]

Antibodies

For a complete list of the applied antibodies, please refer to the Supplementary Table 1.

Immunohistochemistry (IHC), immunofluresence (IF), histopathological evaluation & quantitative assessment of neuro-immunoreactivity in human specimens

The degree of neural invasion and pancreatic neuritis on each nerve was classified as no neuritis/score 0, peri-neuritis/I and endo-neuritis/ II or as no invasion/0, perineural invasion/I or endoneural invasion/II, as also described previously.[1] In particular, neuritis was classified as peri-neural (score I) if inflammatory cells were detected solely in contact with epineurium, and as endo-neural (score II), if inflammatory cells were also present in the endoneurium of nerves.[1] The concentration of the primary antibodies in the immunohistochemistry were as follows: hypoxia-inducible factor-1α (HIF-1α, 1:300 dilution for IHC and IF, rabbit polyclonal, Santa Cruz, Heidelberg, Germany), carbonic anhydrase IX (CA-IX, 1:1000 dilution for IHC, 1:500 for IF, rabbit polyclonal, Abcam, Cambridge, UK), GFAP (1:400

dilution for IHC and IF, rabbit polyclonal, DAKO, Hamburg, Germany), protein gene product 9.5 (PGP9.5, 1:1000, rabbit polyclonal, DAKO, Hamburg, Germany), S100 (1:200, mouse monoclonal, Millipore, Billerica, USA) and ki67 (1:100, mouse monoclonal, DAKO, Hamburg, Germany). In the correlation analysis involving neural GFAP, HIF-1α and CA-IX, 10 randomly selected nerves per patient were analyzed. In the analysis of Schwann cell proliferation within nerves, all visible nerves from 10 patients with PCa or 10 patients with NP were photomicrographed, and the number of $ki67^+$ nuclei within S100 Schwann cells within nerves were proportioned to the total number of nuclei in each nerve. Two different sections that were at least 30 um apart were analyzed from each patient.

Assessment of hSC hypertrophy in PCa- and CCa-conditioned media

hSC were seeded at 5,000 cells/well on poly-D-lysine-coated (40 mg/m^2, Sigma-Aldrich, Taufkirchen, Germany) 13-mm coverslips in 24-well plates (NUNC, Langenselbold, Germany) and grown in their routine complete medium for 24 hours. The medium was then replaced with 500µl serum-free SC medium supplemented with the amount of conditioned supernatant containing 50µg of protein, thus equaling a final concentration of 100µg of protein per ml medium in each well. The supernatants from each cell line were added to three wells per experiment. Each experiment was repeated three times. After 24 hours of supernatant treatment, the cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline, immunostained with a monoclonal antibody against the glial intermediate filament GFAP (GFAP; 1:400, DAKO, Hamburg, Germany), followed by second antibody immunolabeling with Alexa Fluor® Goat anti-rabbit IgG antibodies (1:400 dilution, Invitrogen, Karlsruhe, Germany). The nuclei were visualized by using 4', 6-diamidino-2-phenylindole (DAPI) counterstain (1:400 dilution, Invitrogen, Karlsruhe, Germany). The mean cellular area was calculated by measuring the total GFAP-immunoreactive hSC area in

4 random areas of each coverslips via the threshold function of the ImageJ software (Version 1.44p, Wayne Rasband, NIH; expressed in μm^2) and dividing it by the number of hSC.

Effect of hypoxia upon intermediate filament expression by hSC

To investigate the effect of hypoxia upon GFAP, Nestin and Vimentin production by hSC, sister clones of the hSC were incubated under normoxic and hypoxic conditions (89.25% N_2 + 10% CO_2 + 0.75% 0_2) after reaching 80% confluence for varying time periods, beginning with 15 minutes and gradually increasing to 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours and up to 48 hours at 37°C in serum-free SC medium. At the end of each respective period, the supernatants were collected and the cells were lysed with radioimmunoprecipitation (RIPA) buffer containing Complete, a protease inhibitor cocktail (Roche, Penzberg, Germany). The protein concentration of the whole cell lysates was measured with the BCA protein assay and subsequently subjected to immunoblot analysis.

Multiplex immunoassay

Bio-Plex Pro 27 Plex Human Cytokine, Chemokine and Growth Factor Assay (Bio-Rad Labororatories Ltd, Hercules, CA, USA), on the Bio-Plex 200 System, with initial data analysis performed using Bio-Plex Manager 5.0 Software was used for the measurement of cytokines (platelet-derived growth factor/PDGF, IL1-beta, IL1R-A, IL-4, IL-6, IL-8, IL-9, IL-10, IL-12, IL-13, IL-17, basic fibroblast growth factor/bFGF, granulocyte colony stimulating factor/G-CSF, interferon-gamma/IFN-γ, C-C motif ligand 2/CCL2, monocyte chemotactic protein-1/MCP-1, tumor necrosis factor alpha/TNF-α and vascular endothelial growth factor/VEGF) within serum-free supernatants of hypoxic and normoxic hSC according to the specifications of the manufacturer.

Blocking antibody treatment of hSC and PCC supernatants against IL-6 and IL-1β

To elucidate the impact of IL-6 or IL-1β in hypoxia- and PCC- induced hSC activation, mouse IgG1 anti-human neutralizing antibodies were added at 10 µg/ml each (corresponding to 3xIC50) to the growth medium of hSC under hypoxia (24 h) or normoxia or to the PCC supernatants. Non-immunized mouse IgG1 antibody (Sigma-Aldrich, Taufkirchen, Germany) was added at 10 µg/ml as negative control.

Growth assay and intermediate filament expression in conditioned media

To assess human Schwann cell (hSC) growth, the MTT (3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) assay was used as published before.[4] Briefly, cells were seeded at a density of 5,000 cells/well in 96-well plates, and after 24 hours, their growth medium was supplemented with the volume of PCa- or CCa-cell-conditioned supernatant containing 20µg of protein in a total of 200µl of serum-free SC medium, thus equaling a final concentration of 100µg of protein per ml medium in each well, as described previously.[5] Equal volume of serum-free RPMI-1640 medium without previous contact to cancer cells was used as negative control. The viability was measured at 0 hour, 24 hours (h), 48 hours, 72 hours, 5 days and 7 days after addition of supernatants. For this purpose, MTT was added to each well (50µg/well) and allowed to incubate for 4 hours. Formazan products were solubilized with acidic isopropanol, and the optical density was measured at 570 nm.

In additional experiments, the proliferation rate of hSC exposed to PCa cell supernatants was assessed *in vitro* via double-immunolabeling against ki67 (1:100, mouse monoclonal, DAKO, Hamburg, Germany), S100 (1:200, mouse monoclonal, Millipore, Billerica, USA) or after pre-treatment with bromodesoxyuridine (BrdU) and immunostaining against BrdU (1:100, rat, Santa Cruz Biotechnology, Heidelberg, Germany).

To assess the expression of the intermediate filaments GFAP, Nestin and Vimentin in hSC, 75,000 hSC/well were seeded in poly-D-lysine-covered six-well plates. After 24h of cultivation in routine complete SC medium, the medium was replaced with serum-free SC

medium together with conditioned supernatant containing 100µg of protein per ml medium in each well. Equal volume of serum-free RPMI-1640 medium without previous contact to cancer cells was used as negative control. Serum-free SC medium supplemented with nerve growth factor (NGF, 10ng/ml) was used as positive control due to the previously described activating properties of NGF on SC.[6] hSC were lysed for immunoblot analysis after 48 hours of treatment with conditioned media. All experiments were made in triplicates and repeated 3 times.

BrdU labeling

To assess hSC proliferation under the influence of PCC supernatants, BrdU (Sigma-Aldrich, Taufkirchen, Germany) was added to the serum-free growth medium of hSC at an end concentration of 10µg/ml after 8 hours of PCC supernatant exposure. After 24 hours, hSC were fixed with 4% paraformaldeyhde and immunostained with a BrdU antibody and DAPI. The proportion of BrdU+ hSC was proportioned to the total number of DAPI-stained nuclei on photomicrographs of four randomly selected areas on each coverslip.

Co-culture

For co-culture analysis, 75,000 hSC/well were seeded in poly-D-lysine-covered six-well plates. After 24h of cultivation in routine complete SC medium, a co-culture insert (BD Falcon^{TM}, NJ, USA) containing 100,000 cancer cells, 100,000 (Jurkat)-T-lymphocytes or a 1:1 combination of each (50,000 cancer cells plus 50,000 Jurkat cells) in serum-free RPMI-1640 medium was placed into the wells. After 24 and 48 hours, hSC were lysed with radioimmunoprecipitation (RIPA) buffer containing Complete, a protease inhibitor cocktail (Roche, Penzberg, Germany), and measured for their protein concentration.

Assessment of mechanical abdominal hyperalgesia and open-field behaviour

The extent of reaction to each tactile von Frey stimulus (filament strengths: 0.008, 0.02, 0.04, 0.07, 0.16, 0.4 and 0.6 g/matter) was scored as no reaction (score 0), mild reaction (score 1, e.g. licking of the abdomen), or strong reaction (score 2, e.g. abdominal retraction or jumping). The frequency of each reaction type was multiplied with the corresponding reaction score, and the obtained results were added to generate the "von Frey score" of each animal.

For the open field analyses, mice were allowed to get accustomed to the open field for 10 minutes and then video-recorded for three minutes five consecutive times. The video recordings were analyzed via Mtrack2 plugin of the ImageJ software for the total distance travelled by each mouse in the open-field.

RT^2 Profiler^{TM} polymerase chain reaction (PCR) array

The transcriptional profile of the hSC under hypoxia and normoxia was compared within the total RNA via the RT^2 Profiler^{TM} PCR Array Human Pain: Neuropathic & Inflammatory (SABiosciences, Qiagen, Hilden, Germany) according to the instructions of the manufacturer. The PCR plates were analyzed on the Roche LightCycler® 480 system. The statistical comparisons were performed on the online data analysis center of the manufacturer. The changes in the expression profile were considered relevant for a gene if an absolute fold regulation of at least 2-fold was detected after t-test based statistical comparison of the reference group (RNA of normoxic hSC) with the experimental groups (RNA of hypoxic hSC).

References for supplementary methods

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