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Abstract

As canine mammary tumours (CMT) and human breast cancer share clinical and prognostic features, the former have been proposed as a model to study carcinogenesis and improved therapeutic treatment in human breast cancer. In recent years, it has been shown that transient receptor potential vanilloid 1 (TRPV1) is expressed in different neoplastic tissues and its activation has been associated with regulation of cancer growth and progression. The aim of the present research was to demonstrate the presence of TRPV1 in human and canine mammary cancer cells, MCF-7 and CF.41, respectively, and to study the role of TRPV1 in regulating cell proliferation. The images obtained by Western blot showed a signal at 100 kDa corresponding to the molecular weight of TRPV1 receptor. All tested TRPV1 agonists and antagonists caused a significant decrease ($P < 0.05$) of cell growth rate in MCF-7 cells. By contrast, in CF.41 cells capsaicin and capsazepine induced a significant increase

($P < 0.05$) in cell proliferation, whereas resiniferatoxin (RTX) and 5-iodo-resiniferatoxin (5-I-RTX) had

no influence on CF.41 cell proliferation. Further studies are needed to elucidate the underlying molecular mechanism responsible for the different effects evoked by TRPV1 activation in MCF-7 and CF.41 cells.

Keywords

CF.41, MCF-7, proliferation assays, TRPV1, Western blotting

Introduction

Recent epidemiological data suggest that in 2008 almost 1.4 million women worldwide were diagnosed with breast cancer and approximately 459 000 deaths were recorded. The 5-year relative survival rate ranges from 12 to 90% depending on diagnostic and therapeutic approach.1 Similarly to humans, mammary glands are the most frequent site of neoplasia in female dogs and in this species mammary tumours represent one of the leading causes of death due to development of metastases in regional lymph nodes and lung.2 Clinical and molecular similarities between canine mammary tumours (CMT) and human breast cancer have been described in recent years.3 Interestingly, beside the fact that the etiopathogenesis of CMT is still unclear, by comparing breast cancer frequencies of dog and pet ownership a significant positive correlation was found.4 Indeed, the use of dogs spontaneously affected by cancer in trials testing novel anticancer therapies for humans, is growing up.5,6 As in humans, age, obesity and diet seem to increase the risk of CMT.7 Moreover, inbothspecies prognosis depends on tumour size, histology type, proliferation rate, lymph node status, hormonal receptor status, presence of metastases and gene expression profile.8

The transient receptor potential vanilloid 1 (TRPV1) is a member of the transient receptor potential (TRP) family known to be expressed in several neural and non-neuronal tissues of dif- ferent animal species.9 TRPV1 is a non-selective cation channel that can be activated either by physical, chemical and pro-inflammatory stimuli such as noxious heat (>43 ◦C), low pH (<6) or by different lipophilic compounds including capsaicin and phospholipid derivatives.10 The acti- vation of TRPV1 receptors causes calcium influx and the corresponding release of tachykinin neuropeptides which, in turn, trigger a tissue response known as 'neurogenic' inflammation.11 In recent years, TRP channels have been associated with cancer growth and progression.12 Their expres- sion has been demonstrated in different neoplastic tissues such as squamous cell carcinoma of the human tongue,13 human prostate carcinoma,14 colon cancer,15 transitional carcinoma of human bladder16 and human breast cancer.17 Santoni et al.16 found that TRPV1 expression decreases during the development of transitional cell car- cinoma of human bladder and outlined its key role in the regulation of tumour cell proliferation and even as possible therapeutic target.16 Mammalian TRPV1 receptors were cloned and characterized in humans,18 rats,19 guinea pigs,20 rabbits,21 mice22 and dogs.23 The various TRPV1-homologues share a conserved structure composed by six transmem- brane spanning domains (S1 – S6), intracellular N and C terminals and a pore-forming hydrophobic stretch between the fifth and the sixth domains. TRPV1 receptors are activated by vanilloids-like capsaicin. However, TRPV1 proteins do not always exhibit the same pharmacological profile. While in rat 12-phenyl-acetate 13-acetate 20-homovanillate (PPAHV) acts as a potent agonist for TRPV1, in humans and guinea pig TRPV1s are not stimulated by PPAHV.24,25

As mentioned previously, the canine model is currently used in comparative oncology as spontaneous tumours in dogs share a wide variety of epidemiological, biological and clinical features with human cancers, which make this animal model attractive in oncology research.26 In past decades, dog was studied as a model for prostate tumour,27 osteosarcoma,28 lymphoma29,30 and mammary tumour.26,31 Considering that TRPV1 is expressed both in human and canine species and it is expressed in human breast cancer, the aim of the present research was to study the expression of TRPV1 receptor in cells derived from human breast cancer and canine mammary cancer. Moreover, the pharmacological characteristics of different specific TRPV1 agonists and antagonists were studiedthroughproliferationassays inorderto evaluate the capacity of TRPV1 ligands to modulate the growth of tumour cells.

Materials and methods

Cell culture

MCF-7 cells (cells derived from pleural effusion after pleural methastatization of human breast adenocarcinoma, ATCC cell culture, LGC Stan- dards, Milan, Italy) and CF.41 cells (cells derived from canine mammary adenocarcinoma, ATCC cell culture, LGC Standards, Milan, Italy) were cultured in 75 cm3 flasks containing Dulbecco's Modified Eagles Medium (DMEM, Lonza, Basel, Switzerland) supplemented with 20% Fetal Bovine Serum (FBS, Sigma Aldrich, Milan, Italy), 2% penicilllin– streptomicin– amphotericin B solution (Sigma Aldrich) and 2% L-glutamine (Sigma Aldrich) at 37 ◦C with 5% CO2, 95% air and com- plete humidity. When reaching a confluence of 80%, cells were detached using 0.1% Trypsin/EDTA solution (Sigma Aldrich), centrifuged and counted using Trypan Blue solution (Sigma Aldrich) with a Burker chamber. Cells were then resuspended at a concentration of 5 × 105 cells mL−1 and seeded at the same conditions mentioned before.

Western blotting assay

Thawed cells (concentration 180 × 106 cells approx- imately), re-suspended in binding buffer (KCl 5 mM, NaCl 5.8 mM, CaCl2 0.75 mM, MgCl2 2 mM, sucrose 320 mM and Hepes 10 mM, pH 7.4, Sigma Aldrich), were disrupted using a soni- cator (XL 2020, Misonix, Farmingdale, NY, USA) and centrifuged at 3000 × g for 10 min at 4 ◦C. The resulting surnatants were ultra centrifuged at 105 000 × g for 45 min at 4 ◦C to separate

cytoso- lic fraction from cell membrane fraction. The final pellets were suspended in the same buffer and the protein concentration was measured according to the method described by Lowry et al.32 Samples were loaded at 25, 50 and 100 □g mL−1 of pro- tein per lane and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS- PAGE) procedure using 8% Acrylamide (Biosolve, Valkenswaand, The Netherlands) gel. A proteic lysate of urinary rat bladder was used as positive control. Samples were then transferred to nitro-cellulose membranes (Hybond ECL, Amersham, GE Healthcare, Milan, Italy). After being washed three times, samples were probed with the primary anti-TRPV1 antibody (goat polyclonal antibody, Santa Cruz Biotechnology, Heidelberg, Germany) (dilution 1:100) and incubated overnight at 5 ◦C on a shaking plate. At the end of the incubation period samples were washed again for four times and probed with the secondary antibody (donkey anti-goat IgG– HRP, Santa Cruz Biotechnology) (dilution 1:15 000) for 1 h. Secondary antibody was removed by fourwashes andimmunoreactive bands were visualized by enhanced chemiluminescence (ECL-Plus System, Amersham, GE Healthcare). To obtain an endogenous control, membranes were washed and re-probed with β-actin antibody (dilu- tion 1:100, Santa Cruz Biotechnology) for 1 h. Also, in this case the immunoreactive bands of β- actin were visualized by ECL (ECL-Plus System, Amersham, GE Healthcare).

Proliferation assays

3-(4,5-Dimethylthiazol-2-yl)-2,5,-dipheyltetrazo- lium bromide (MTT, Sigma Aldrich) reduction assay was performed for both cell lines. This method is one of the most frequently used for quantitating cell viability and it measures the conversion of MTT into purple-coloured formazan that is induced by redox activity of living cells: a decrease of cellular redox activity could be index of cell damage.33

Cultured cells were detached using 0.1% Trypsin/EDTA solution (Sigma Aldrich) and diluted to a final concentration of 5 × 103 cells 100 μL−1. The cell suspension was trans- ferred into 96-well plates with DMEM (Lonza, Basel, Switzerland), 10% FBS (Sigma Aldrich), 2% penicilllin– streptomicin– amphotericin B solu-tion (Sigma Aldrich) and 2% L-glutamine (Sigma Aldrich). Each drug was tested in two different experimental sessions and three plates were pre- pared per each experimental session (n = 6). To evaluate the background adsorption, the first row of every plate remained cell-free as blank control.

In the second row, cells were seeded in absence of any drug as internal control. The remaining part of the plates was used to test TRPV1 ligands. After an overnight incubation at 37 ◦C and 5% CO2, the medium used for the seeding was replaced by a medium prepared with DMEM, 5% FBS, 2% penicilllin– streptomicin–amphotericin B solution, 2% L-glutamine with the addition of increasing con- centrations of the following TRPV1 ligands: Cap- saicin and capsazepine (partial agonist and antago- nist, respectively, Sigma Aldrich) (10 − 10 –10−4M), resiniferatoxin (RTX) and 5-iodo-resiniferatoxin (5-I-RTX) (pure agonist and antagonist, respec- tively, Sigma Aldrich) (10−10 –10−6M). Plates were incubated at 37 ◦C at 5% CO2 for 24, 48 and 72h. At each experimental time point, 20 μL of MTT (Sigma Aldrich) solution (4 mg mL−1) were added to each well, except to the free-cell blank wells, and plates were incubated at 37 ◦C and 5% CO2 for 4h. At the end of the incubation period, the medium containing the MTT solution was discarded and 100 μL of dimethyl sulfoxide (DMSO, Sigma Aldrich) were added in order to dissolve the formazan crys- tals. The plates were further incubated for 10 min at room temperature and then read using a plate reader (Powerwave X, Bio-Teck, Winooski, VT, USA) at a wavelength of 570 nm.

The results obtained by the proliferation assays were statistically analysed using a computer programme (GraphPad Prism Software, San Diego, CA, USA), applying the Kruskal– Wallis test and a Dunn's post-test ($P < 0.05$).

Results

Western blotting

Western blotting analysis indicated that the TRPV1 protein is expressed in MCF-7 cells as shown by the high density signal corresponding to the molecular weight of 100 kDa, in accordance with the mass of human TRPV1 (Fig. 1A).34 The signal corresponding to 100 kDa was also recognizable in the Western blotting analysis of CF.41 cells (Fig. 1B). The same signal was present in positive control (lysate of rat urinary bladder). Fig. 1C shows the signal corresponding to β-actin, used as endogenous control.

Proliferation assays

MCF-7 proliferation assay data are summarized in Figs 2– 5. All tested agonists and antagonists induced a significant decrease ($P < 0.05$) of growth rate in treated cells compared with controls. RTX showed a characteristic time-dependent behaviour (Fig. 4). As far as CF.41 cells are concerned results are presented in Figs 6– 9. The partial agonist (capsaicin) and the partial antagonist (capsazepine) caused a significant increase ($P < 0.05$) of CF.41 cell proliferation. By contrast, RTX and 5-I-RTX did not induce any significant effect on cell growth rate.

Discussion

To our knowledge, this is the first study demon- strating the expression of TRPV1 in CF.41 cells, while TRPV1 in MCF-7 cells had already been identified by Barbero et al.35 in 2006. Increased expression of TRPV1 is associated with a better prognosis of patients with hepatocellular carci- noma, whereas expression of TRPV1 is decreased in the urothelium of patients with advanced stage transitional cell carcinoma, suggesting that TRPV1 might function as a tumour suppressor and nega- tively control tumour progression.36 Different TRP channels are also expressed in human breast can- cer cells and cell lines such as MCF-7 and their overexpression compared to normal gland can be considered a suitable prognostic marker.37 It has been suggested that the receptor-mediated anti-tumour activity is associated with induction of apoptosis following activation of TRPV1 and changes in cell calcium influx.38 Calcium is an important intracellular messenger that regulates many physiological functions such as cellular dif- ferentiation, proliferation and apoptosis. Abnor- mal functioning of calcium signalling pathways may occur during cancerogenesis. Therefore, cal- cium channels with increased expression and/or activity in cancer could potentially serve as anti- cancer therapeutic targets. Recent investigations have suggested that besides proapoptotic action, the activation of TRPV1 leads to anti-angiogenic, anti-migrative, anti-adhesive and anti-metastatic effects.39 As TRPV1 is implicated in cell pro- liferation and pain modulation, a number of studies have been performed to describe the bind- ing characteristics and clinical efficacy of selective TRPV1 agonists and antagonists.40 Capsaicin and RTX are selective TRPV1 agonists and cause TRPV1 desensitization after activation while cap- sazepine and 5-iodo-resinferatoxin are selective TRPV1 antagonists inducing a functional block of TRPV1 receptor.41 In our study, all tested ago- nists and antagonists caused a significant decrease ($P < 0.05$) in MCF-7 cell proliferation. By contrast, capsaicin and capsazepine induced a significant increase ($P < 0.05$) in CF.41 cell proliferation. These findings might account for species-specific differences in TRPV1 pharmacological proper- ties. In rodents, TRPV1 is activated by capsaicin and residues Arg114 and Glu761 in the intra- cellular N- and C-termini have been recognized as agonist binding sites.42 Unlike the murine one, the avian form of TRPV1 is not activated by capsaicin and the agonist binding sites are represented by residues Tyr511 and Thr550 in the third and fifth transmembrane domains.21 Moreover, species-dependent behaviour has been observed also for antagonists. According to Premkumar and Sikand,43 the TRPV1 competitive antagonist capsazepine inhibits the acid-induced activation of human TRPV1, but exhibits no effects on acid-induced activation of rat TRPV1. The compound can inhibit heat-induced TRPV1 activation both in humans and rat. Besides this, it has been demonstrated that TRPV1 mutations can lead to changes in TRPV1 responses to agonist stimulation. Mutations at the sixth TRPV1 transmembrane domain can cause, for example, the reduction of 3[H]resiniferatoxin-binding affinity and mutations on pore domain significantly decrease the sensitivity to capsaicin.44 A further hypothesis to explain the different effects induced by TRPV1 agonists and antagonists on MCF-7 and CF.41

proliferation might be the pos- sible interaction of capsaicin and capsazepine with other TRP or non-TRP receptors. Hwang et al.41 found that capsaicin might act as co-carcinogen on 12-O-tetradecanoylphorbol-13-acetate (TPA)- induced skin carcinogenesis through the epidermal growth factor receptor. As the MTT proliferation assay does not provide information concerning the molecular mechanisms involved in TRPV1 responses, further studies are needed to under- stand differences in human and canine breast cancer cell proliferation upon TRPV1 agonist and antagonist stimulation. The results of proliferation assays performed on MCF-7 confirm the potential of TRPV1 agonists as anti-cancer drugs. Interest- ingly, capsaicin was shown to inhibit human cancer hormone-resistant cells and to provide chemopre- ventive effects.45 Moreover, it was demonstrated that capsaicin is able to mediate cell death of T24 (human bladder cancer) and A172 (human glioma) cell lines.46,47 Even if the results of proliferation assays on MCF-7 cells seem to suggest that also TRPV1 antagonists could be used in cancer treat- ment, their use should be carefully considered: the systemic administration of TRPV1 antagonists could lead to the change of body temperature, gas- trointestinal and cardiovascular functions.43 Fur- thermore, the blockade of TRPV1 has been shown to increase its own expression, thereby raising the possibility of rebound effects.43 Therefore, TRPV1- mediated effects onhuman and canine breast cancer cells should be encouraged.

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