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Xanthohumol Inhibits Inflammatory Factor Production and Angiogenesis in Breast Cancer Xenografts

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Abstract Xanthohumol (XN), a natural polyphenol present in beer, is known to exert anti-cancer effects. However, its precise mechanisms are not yet clearly defined. The aim of this study was to investigate the effect of oral administration of XN in breast cancer xenografts in nude mice. Proliferation and apoptosis were first examined in MCF7 cell cultures after incubation with XN by trypan blue exclusion assay, [^{3}H]-thymidine incorporation, KI67 immunostaining and TUNEL. Morphological and histological characteristics of tumours from XN-treated or control (vehicle-treated) mice were compared. Immunohistochemistry for proliferative, inflammatory and endothelial cell markers was performed and activation of nuclear factor kappa B (NFκB) pathway was assessed by ELISA. In vitro MCF7 cell proliferation decreased in a dose-dependent manner. Oral administration of XN to nude mice inoculated with MCF7 cells resulted in central necrosis within tumours, reduced inflammatory cell number, focal proliferation areas, increased percentage of apoptotic cells and decreased microvessel density. Anti-angiogenic effects of XN were further confirmed by immunoblotting for factor VIII expression in XN-treated tumours as compared to controls. Decreased immunostaining for NFκB, phosphorylated-inhibitor of kappa B and interleukin-1β were also observed as well as a significant decrease in NFκB activity to 60% of control values. These novel findings indicate that XN is able to target both breast cancer and host cells, namely inflammatory and endothelial cells, suggesting its potential use as a double-edge anti-cancer agent. J. Cell. Biochem. 104: 1699–1707, 2008. © 2008 Wiley-Liss, Inc.

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Current estimates indicate that dietary factors contribute to one-third of annual cancer-related deaths in the United State. In contrast to a typical western diet, consumption of plant-derived foods offer a protective effect against cancer [Rowland, 1999; Le Marchand, 2002; Lambert et al., 2005]. Therefore, an important strategy for cancer prevention is the identification and characterization of dietary phytochemicals that are able to block, slow or reverse carcinogenesis. Polyphenols are one of the largest and ubiquitous groups of phytochemicals to which anti-cancer properties are being increasingly attributed.

Recently, much attention is being drawn over xanthohumol (XN), a prenylated flavonoid isolated from hops (*Humulus lupulus* L.), that possesses a large spectrum of chemopreventive mechanisms in a wide variety of cancer cell lines [Miranda et al., 1999; Gerhauser et al., 2002; Gerhauser, 2005; Lust et al., 2005; Monteiro et al., 2007]. Accordingly, XN has been reported to modulate pro-carcinogen activating and detoxifying enzymes, besides exhibiting anti-oxidant and free radical-scavenging activity

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[Miranda et al., 2000; Stevens et al., 2003; Gerhauser, 2005; Lust et al., 2005]. XN significantly reduces proliferation and activates caspase cascades in human colon cancer cells, implying antiproliferative and apoptotic effects [Pan et al., 2005]. This compound is also regarded as an anti-inflammatory and antiangiogenic agent, by abrogating the expression of several inflammatory genes, such as cyclo-oxygenase (COX)-1, COX-2 and inducible nitric oxide synthase [Gerhauser et al., 2003].

Breast cancer is the most frequently diagnosed cancer among women and the second leading cause of cancer-related deaths [Levi et al., 2005]. Some effects of XN have previously been reported in different breast cancer cell lines. For example, XN inhibits growth and induces cytotoxicity in human MCF7 and SKBR3 [Miranda et al., 1999; Monteiro et al., 2007]. Nevertheless, most of the studies concerning the role of XN as an agent against breast cancer were performed in vitro. Given the wide variety of effects of XN and the relevant interaction between tumour cells and host neighbouring cells in cancer progression, it is imperative to elucidate the underlying mechanisms of XN on breast cancer in vivo.

MATERIALS AND METHODS

Cell Culture

Estrogen-dependent human breast cancer MCF7 cells (American Type Culture Collection, Rockville, MD) were maintained in minimum essential medium (MEM; Gibco BRL, Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Life Technologies). For every experiment, cells were incubated with XN (kindly provided by Instituto de Bebidas e Saúde, iBeSa, Portugal) in cell medium containing 5% FBS. Control cells were incubated with ethanol at a concentration of less than 0.1%.

Trypan Blue Exclusion Assay

Cells were treated with XN at concentrations of 0.1, 1.0, 10, 50 and 100 μM or ethanol for 24 and 72 h. Viability was evaluated by counting viable and dead cells in a haemacytometer. The cytotoxic effect of XN was calculated as described earlier [Blishchenko et al., 2002].

[^{3}H]-Thymidine Incorporation

MCF7 cells previously incubated with distinct concentrations of XN for 24 and 72 h, were treated with methyl-[^{3}H]-thymidine (0.5 μCi/well) in cell medium for 4 h. Cells were then fixed in 10% trichloroacetic acid (TCA) for 1 h at 4°C, washed twice with 10% TCA to remove unbound radioactivity, air-dried and lysed with 1 M NaOH (0.28 ml/well). Methyl-[^{3}H]-thymidine incorporated into cellular DNA was quantified by liquid scintillation counting.

In Vivo Studies

Animal experiments were conducted according to accepted standards of human animal care (European Community guidelines (86/609/EEC) and Portuguese Act (129/92) for the use of experimental animals). Ten male nude mice (N: NIH (s) II strain) 4–6 weeks old, were housed in a pathogen-free environment under control conditions of light and humidity. Mice were subcutaneously implanted with a 25 μg/day release 17β-estradiol pellet (Innovative Research of America, USA), the day before cell inoculation. Mice were then inoculated with 5×10^7 MCF7 cells in the mammary fat pad and divided into two groups: control group—ad libitum ingestion of water with vehicle (0.1% ethanol) and a xanthohumol-treated group—ad libitum ingestion of 100 μM xanthohumol solution for 60 days. Drinking solutions were renewed every other day and were kept in dark bottles to avoid degradation. Food and fluid consumption, as well as body weight and tumour volume were monitored weekly throughout the experiment.

Tissue Collection and Preparation

At the end of the experiment, animals were euthanised and tumours were removed, weighed, formalin-fixed and paraffin-embedded. Histological, immunohistochemical or apoptosis analyses were then assessed in 4-μm tissue sections. Tumour histology was observed and evaluated on hematoxylin-eosin (HE)-stained sections.

Immunohistochemistry Analyses

Expression of nuclear factor kappa B, phosphorylated (Pi) inhibitor of kappa B alpha (IκBα), interleukin (IL)1β, the proliferation nuclear marker KI67 and CD31 were analysed

in tumour sections by immunohistochemistry assays using avidin-biotin-peroxidase complex method. KI67 expression was also determined in methanol-fixed MCF7 cells. Antibodies against inflammatory markers and KI67 were purchased in Santa Cruz Biotechnologies, CA. Immunostaining for CD31 (Novocastra, UK) was preceded by pepsin digestion of the samples at room temperature for 30 min. Negative controls were carried out by omission of the primary antibody and sections of tissues known to express each marker were used.

CD31-positive microvessels were counted in the three most vascularized areas of tumour sections in a 200× field (0.74 mm^2) by four observers simultaneously [Soares et al., 2004]. Any positive single cell or cluster of cells stained, clearly separated from adjacent clusters and background, with or without lumen, was considered an individual vessel.

Apoptosis Assay

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling assay (TUNEL) (Roche Diagnostics, Basel, Switzerland) was used as previously described [Soares et al., 2004, 2007]. Slides were visualized under a fluorescence microscope (Olympus, BH-2, UK) at a magnification of 200×. Apoptosis was determined as the percentage of positive cells per 1000 DAPI-stained nuclei.

Western Blotting Assay

Proteins were isolated from every tumour using Tripure (Roche Diagnostics, Basel, Switzerland), and quantified by spectrophotometry (Jenway, 6405 UV/vis, Essex, UK). Equal amounts of protein were subjected to 10% SDS–PAGE with a 5% stacking gel. After electrophoresis, proteins were blotted into a Hybond nitrocellulose membrane (Amersham, Arlington), using a mini-transblot electrophoretic transfer cell (Amersham Biosciences). Immunodetection for factor VIII and β-actin (Santa Cruz Biotechnol) was accomplished with enhanced chemiluminiscence (ECL kit, Amersham Biosciences). The relative intensity of each protein blotting analysis was measured using a computerized software program (Biorad, Portugal) and normalized with β-actin bands to compare the expression of proteins in different treatment groups. Experiments were repeated twice.

ELISA Assay

Nuclear extracts were prepared from MCF7 cells or from nude mice tumours using the Nuclear extraction kit (Active Motif, CA). NFκB activity was measured using TransAM NFκB p65/p50 transcription factor assay kit (Active Motif). In brief, nuclear extract samples (5 μg) were added to a 96-well plate with immobilized oligonucleotide containing the NFκB consensus site. Sample wells were incubated with NFκB primary antibody, followed by incubation with HRP-conjugated secondary antibody. Quantification was performed at 450 nm with reference at 650 nm using a plate reader (Thermo Electron Corporation, Multiskan Ascent).

Statistical Analysis

All in vitro experiments were performed in triplicate. Results are expressed as means (SD). Differences between samples were evaluated by Student's *t*-test. Differences were considered statistically significant when $P < 0.05$.

RESULTS

XN Affects MCF7 Cell Proliferation and Cytotoxicity

Treatment of MCF7 cells with xanthohumol (1–100 μM) for 24 h significantly decreased cell proliferation in a dose-dependent manner (Fig. 1A), as determined by the trypan blue exclusion method. At 0.1 μM there was no effect of XN after 24 h of treatment. Cell incubation with XN in the same concentrations for 72 h did also result in significantly decreased total cell numbers (Fig. 1A). Cytotoxic effects were displayed by XN (50 μM) after 24 h of treatment (Fig. 1B).

XN treatment decreased DNA synthesis in every concentration tested (0.1–100 μM) after 24 and 72 h of treatment in a dose-dependent manner, as shown by the decrease in the incorporation of methyl-[^{3}H]-thymidine into DNA (Fig. 2A). The percentage of cells that immunostained for KI67 decreased to 50% of the control values whenever MCF7 cells were incubated with 10 μM XN for 24 h (Fig. 2B).

Effect of XN on Tumour Histological Features

MCF7 cells (5×10^7) were inoculated in nude mice and 100 μM XN or ethanol were provided as the sole drinking source. No significant differences were found between treatment

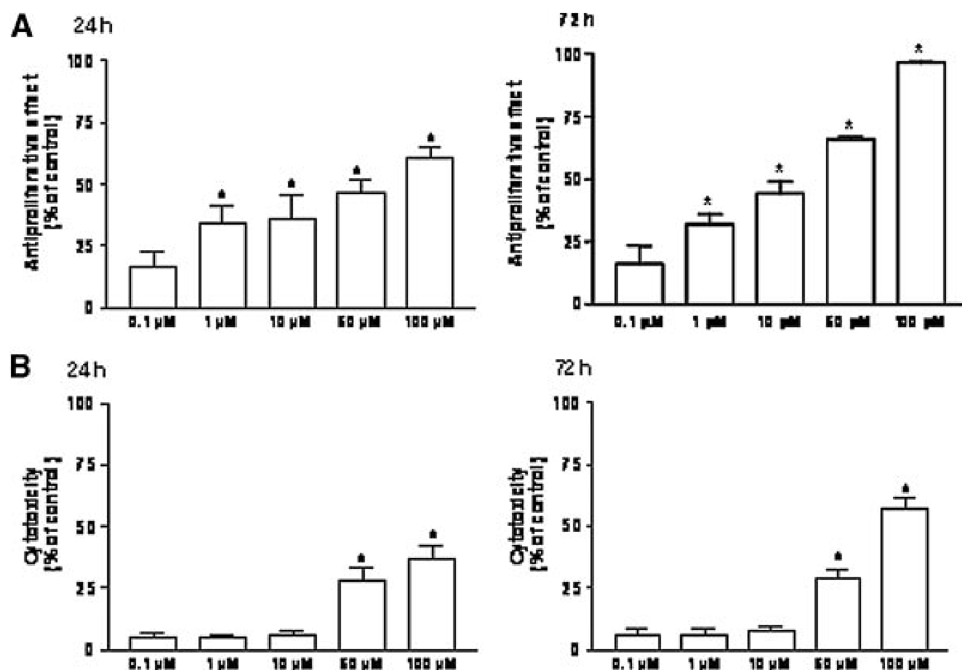

Fig. 1. Effects of xanthohumol (XN) treatment in viability (**A**) and cytotoxicity (**B**) of MCF-7 cells. Cells were treated for 24 and 72 h with different concentrations of XN or vehicle (0.1% ethanol) in culture medium with 5% FBS. Viable cells were counted by the trypan blue exclusion method. Results are means ± SD of three different experiments carried out in duplicate and are expressed as percentage of control. *$P < 0.05$ vs. control.

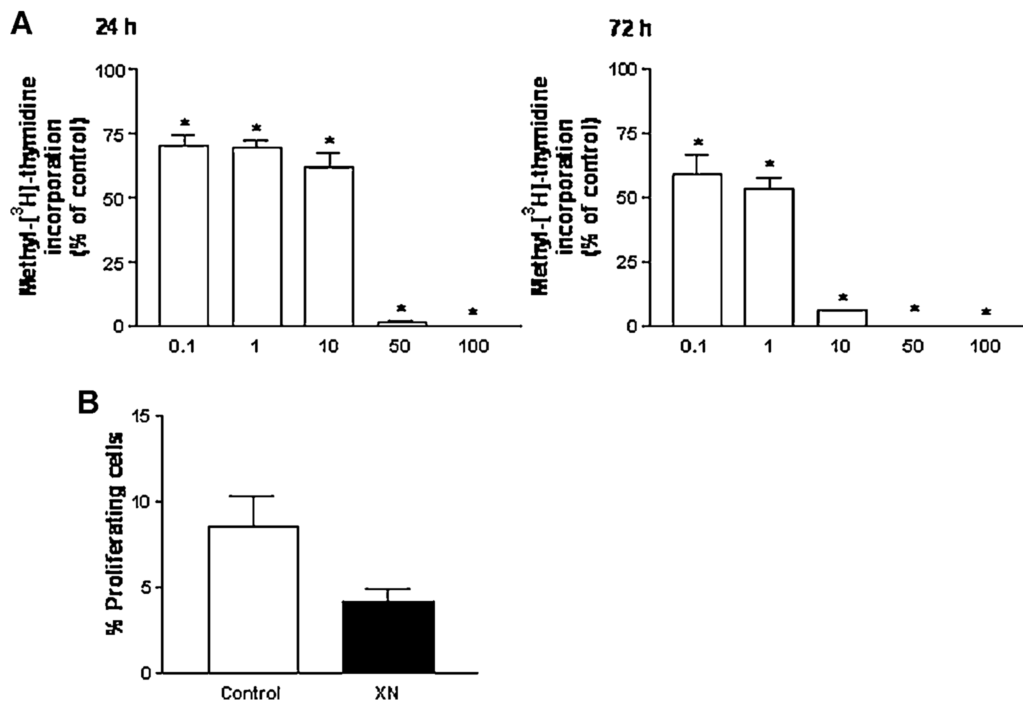

Fig. 2. Xanthohumol (XN) decreased MCF7 cell proliferation. **A**: MCF-7 cells were treated for 24 and 72 h with different concentrations of XN or ethanol in culture medium with 5% FBS. DNA synthesis was significantly decreased by incubation with XN for 24 and 72 h in all concentrations tested. *$P < 0.05$ vs. control. **B**: XN (10 µM) resulted in decreased percentage of KI67-positive cells relative to control (*$P < 0.05$ vs. control). Results are mean ± SD of three independent experiments carried out in duplicate and expressed in percentage of control values.

groups regarding food and fluid intake or body weight throughout the study.

Both groups of mice developed palpable tumours within eight days after inoculation. There was no significant difference in tumour size between the two groups measured during the experiment, although a lower mean weight was found in tumours from XN-treated mice (Fig. 3).

Morphological features were highly reproducible among each group of tumours. XN-treated tumours showed an evident decrease in the number of tumour-surrounding mononuclear and polymorphonuclear inflammatory cells (Fig. 4A), whereas control tumours presented a strong inflammatory infiltrate of both cell types. In addition, XN-treated mice presented large central necrosis, reaching nearly half of the tumour sections (Fig. 4A inset). In contrast, necrosis was not found in any of the control tumours (Fig. 4A).

To further evaluate the distinct inflammatory response, immunostaining for inflammatory factors was investigated. Despite an intense cytoplasmic and nuclear NFκB staining being found in control tumours, no NFκB expression was found in three out of five XN-treated tumours (Fig. 4B). NFκB is prevented to translocate to the nucleus by binding to IκB repressor. Whenever phosphorylated, IκB releases NFκB, which acts as a gene transcription factor in the nucleus [Wang et al., 2002]. Therefore, to confirm whether XN prevents NFkB activity, Pi-IκBα expression was next examined in XN-treated and control tumours. Cytoplasmic staining of Pi-IκBα was abundant in every control tumour, whereas only one XN-treated tumour presented

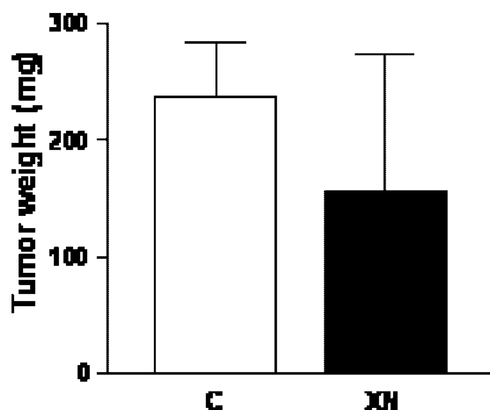

Fig. 3. Effect of xanthohumol in tumour weight. A nonstatistical decrease in tumour weight was found in XN-treated mice as compared to control. Results are means ± SD.

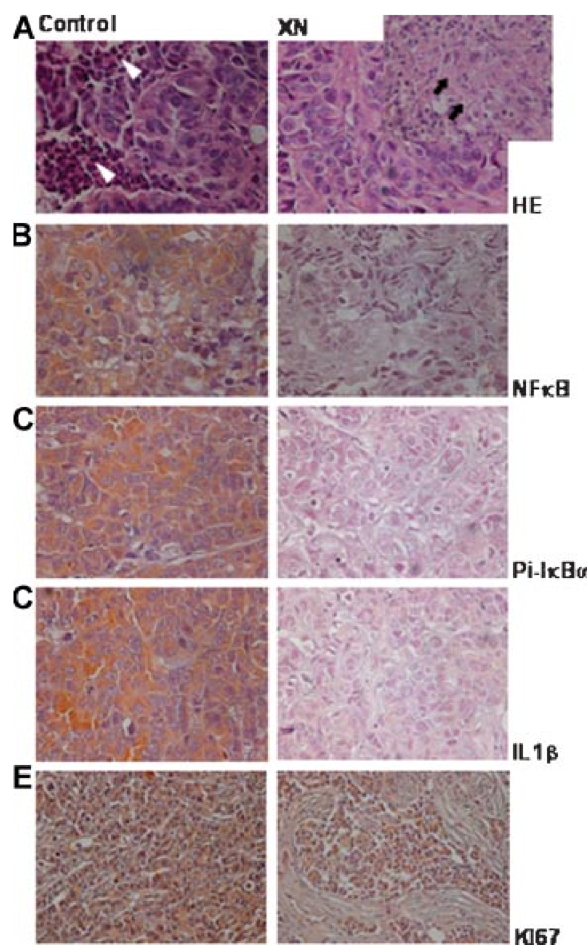

Fig. 4. Micrographs of breast tumour sections from controls or mice receiving xanthohumol (XN). **A**: HE staining showing necrotic area and a decreased number of inflammatory cells within XN-treated tumours (magnification: 200×). Note the increased inflammatory response in the control (arrow heads). Inset shows central necrosis (arrows) found in XN-treated tumours. Immunohistochemical analyses of breast tumour tissue using NFκB (**B**), Pi-IκBα (**C**), IL1β (**D**) (magnification: 400×) and KI67 (**E**) (magnification 200×), Note that immunostaining for KI67 in XN-treated tumours exhibits a focused pattern, whereas a diffuse pattern was observed among control (vehicle) tumours. Intense immunostaining for the three inflammatory markers was observed in every control tumour but not in XN-treated ones. A representative tumour section is shown for each immunostaining. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

a focal moderate intensity Pi-IκBα staining (Fig. 4C). Interleukin1β (IL1β), a cytokine frequently overexpressed in breast cancer (24), presented a multifocal expression in controls but was absent in XN cases (Fig. 4D).

XN Inhibited NFκB Activity in Tumours but not in MCF7 Cells In Vitro

We next investigated whether the reduction in NFκB expression in tumours from XN-treated

mice was accompanied by loss of activity of this transcription factor in MCF7 cells as well as in MCF7 xenografts. Interestingly, XN at 10 μM concentration did not significantly affect NFκB p65 subunit activity as compared to control MCF7 cells in vitro (Fig. 5A). However, tumour cell lysates from XN-treated mice presented a significant reduction of NFκB p65 subunit activity ($P < 0.05$ vs. controls), relative to control tumours (Fig. 5B).

XN Affects Tumour Cell Proliferation, Apoptosis and Angiogenesis

The effect of XN in tumour cell proliferation and apoptosis was then evaluated. The percentage of KI67-stained tumour cell nuclei, did not significantly differ between groups (Fig. 6A). However, in contrast to a diffuse pattern of distribution of proliferating cells in the control cases, tumours from mice receiving XN presented only focal areas of proliferating tumour cells (Fig. 4E). Cell apoptosis was significantly higher in XN-treated tumours than in controls, indicating effective apoptosis by XN in vivo ($P < 0.05$) (Fig. 6B). As XN has been suggested as an anti-angiogenic agent [Albini et al., 2006], tumour microvessel density (MVD) was also evaluated. Microvessels were mainly found at tumour periphery. A decrease in MVD was found in XN-treated tumours as compared to controls (Fig. 6C). To further confirm the inhibitory effects of XN in angiogenesis, the expression of factor VIII, an endothelial marker, was examined by Western blotting. Tumours from mice administered with XN presented significantly decreased levels of factor VIII as compared to control tumours ($^*P = 0.005$ vs. controls) (Fig. 6D), implying that XN actually exhibited anti-angiogenic effects.

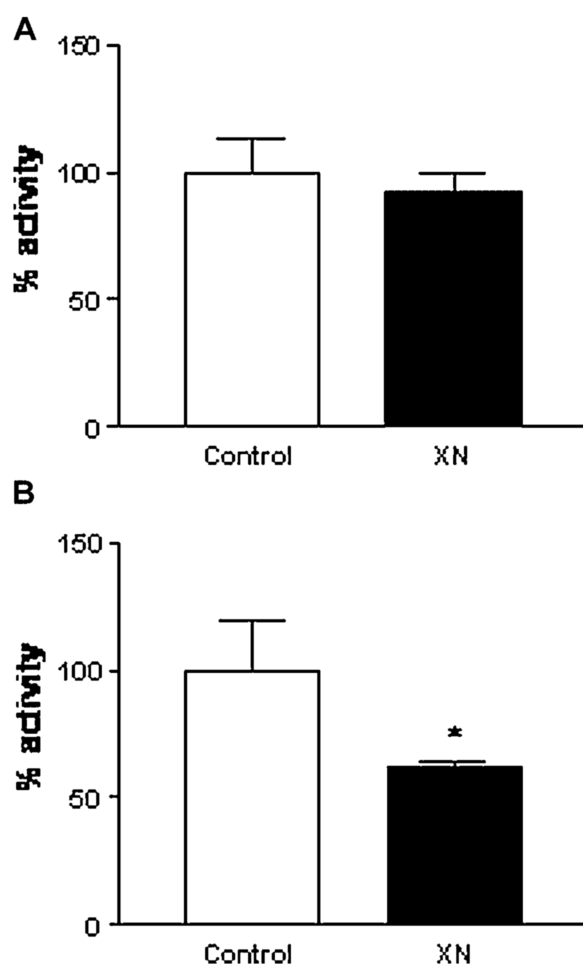

Fig. 5. Effects of xanthohumol (XN) in NFκB p65 subunit activation in MCF7 cells and tumours. **A**: No significant difference in NFκB activation was found between MCF7 cells treated with XN and ethanol (**B**) Tumour cell lysates from nude mice previously treated with XN or ethanol were tested for NFκB activation. A downregulation of NFκB activity was found in tumours from XN-treated mice as compared with tumours from controls ($^*P < 0.05$ vs. control). Equal amounts of protein were loaded. Results are mean ± SD of three independent experiments performed in triplicate.

DISCUSSION

The effects of xanthohumol are being documented in tumour cells [Miranda et al., 1999, 2000; Gerhauser et al., 2002, 2003; Stevens et al., 2003; Gerhauser, 2005; Lust et al., 2005; Pan et al., 2005; Monteiro et al., 2007]. However, not much attention has been paid to cancer neighbouring host cells. These are known to efficiently contribute to cancer progression. Tumour-associated macrophages are a significant component of neoplastic tissues, and are widespread in breast cancer [Kelly et al., 1988; Bingle et al., 2002; Zhao et al., 2003; Stevens and Page, 2004; Colgate et al., 2007].

The current study showed that oral administration of XN to breast cancer-bearing mice resulted in extensive necrosis and increased apoptosis within tumours, but also reduced inflammation and MVD. Accordingly, tumours from XN-treated mice exhibited reduced expression of factor VIII (Fig. 6D), a surrogate endothelial marker. These findings confirm the inhibitory effects of this polyphenol in the angiogenic process.

Previous studies on the Kaposi's sarcoma in mice [Albini et al., 2006], mentioned for the first time anti-apoptotic and anti-angiogenic in vivo effects of XN through NFκB signalling

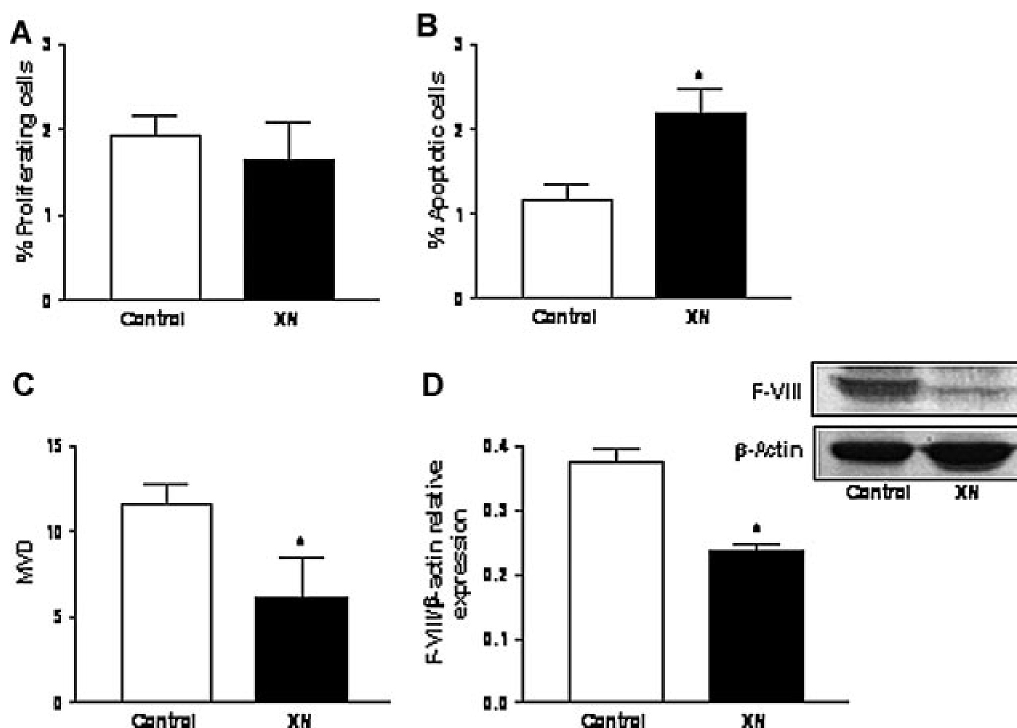

Fig. 6. **A**: Effect of xanthohumol (XN) on tumour cell proliferation revealed by immunohistochemistry. XN led to a decrease in the percentage of KI67-positive cells. Results are mean ± SD of three independent experiments carried out in duplicate. **B**: Tumour cell apoptosis determined by TUNEL assay. XN resulted in a significant increase in apoptosis (*$P < 0.05$ vs. control). Results are means ± SD. **C**: Tumour microvessel density (MVD) in control or XN-treated mice. MCF7 tumours of XN-treated mice presented a significant decrease in the number of MVD in comparison to controls. Results are means ± SD. *$P < 0.05$ versus control. **D**: Expression of factor VIII in tumours from mice previously administered with XN or ethanol (control). A lower expression of factor VIII was found in XN-treated tumours in comparison to controls (*$P = 0.005$ vs. controls). Loading control was confirmed by probing stripped blots for β-actin as shown. A representative Western blotting is shown from two independent experiments.

inhibition. NFκB is an inflammatory promoter also involved in proliferation and down-regulation of apoptosis [Kelly et al., 1988; Magne et al., 2006; Colgate et al., 2007]. These three cellular phenomena were affected by XN in the present study, which led us to hypothesise that NFκB signalling modulation might be underlying the effects of XN in breast tumours. In this regard, we found a significant decrease in NFκB expression and activity in breast tumours from XN-treated mice. Most remarkably, this effect was not observed in MCF7 cultures, indicating that the host tumour environment is probably playing a role, this being also supported by decreased IκBα and IL1β expression in the same animals, the later being usually increased in breast tumours [Hefler et al., 2005].

Altogether, these results demonstrate that XN influences the interplay between tumour cells and host neighbouring cells, probably through the modulation of inflammatory cytokine release. Our findings further stress that besides acting on highly vascularized tumours [Albini et al., 2006], XN also affects low vascularized ones that do not efficiently depend on angiogenesis, as in the case of the presently induced MCF7 xenografts. MCF7 tumours usually maintain a slow growing behaviour, which is related to the absence of tumour burden observed in our experiment as opposite to the Kaposis' sarcoma findings. It has also been demonstrated that breast tumours containing increased numbers of macrophages are significantly more vascularized and metastatic than tumours with a low number of macrophage infiltrates [Bingle et al., 2006]. In accordance, we have found a decrease in MVD, corroborated by an effective reduction of factor VIII immunoexpression. In concert with the decrease in inflammatory factors in XN treated mice, these results support earlier findings that angiogenesis modulation is one of the possible mechanisms for the interference of XN also in MCF7 tumours. Our results

indicate as well that the lack of inflammatory infiltrate in XN-treated group is likely to prevent tumours from developing an angiogenic phenotype.

In summary, our findings demonstrate that XN simultaneously hinders tumour and inflammatory cells and angiogenesis, providing evidence of its interference with tumour-host crosstalk. Despite further studies, with longer experimental periods and more aggressive breast cancer cell lines are needed to determine the chemopreventive and chemotherapeutic effects of XN on human breast cancer in different settings and to better understand its exact mechanisms of action, the potential utility of this polyphenol as a therapeutic tool in breast cancer management becomes emphasised.

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