An ethanolic extract of *Uncaria tomentosa* reduces inflammation and B16-BL6 melanoma growth in C57BL/6 mice.

[Un extracto etanólico de Uncaria tomentosa reduce la inflamación y el crecimiento del melanoma B16/BL6 en ratones C57BL/6]

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Abstract

Extracts of the bark of *Uncaria tomentosa* (Cat’s Claw – Uña de Gato) have been used traditionally for their anti-inflammatory and anticancer properties. We investigated the effect of a hydroethanolic extract (UT) of *U. tomentosa* on a) the viability of primary and tumor cells, b) the inflammatory response (tumor necrosis factor alpha [TNF-α], interleukin-6 [IL-6] and nitric oxide [NO]) both in vitro and in vivo, c) B16/BL6 melanoma cell growth and metastasis in the C57BL/6 mouse, and d) nuclear factor κB (NF-κB) activity in LPS-stimulated HeLa cells. UT did not show an important cytotoxic effect in vitro at the doses up to 300 µg/ml, but did inhibit tumor growth and metastasis in vivo. UT inhibited TNF-α, IL-6 and NO production in vitro. NF-κB activity was also inhibited. Our studies show that UT merits further study for its effects on processes common to inflammation and cancer.

**Keywords:** Uncaria tomentosa, cancer, NF-κB, inflammation, B16/BL6 mouse

Resumen

Los extractos de la corteza de *Uncaria tomentosa* (Uña de Gato) han sido usados tradicionalmente por sus propiedades anti-inflamatorias y antitumoral. Investigamos el efecto de un extracto hidroetanólico (UT) de *U. tomentosa* sobre: a) la viabilidad de células normales y tumorales, b) la respuesta inflamatoria (factor de necrosis tumoral alfa [TNF-α], interleuquina-6 [IL-6] y óxido nítrico [ON]) both in vitro and in vivo, c) B16/BL6 melanoma cell growth and metastasis in the C57BL/6 mouse, and d) actividad del factor nuclear κB (NF-κB) in células HeLa estimuladas con LPS. UT no mostró un efecto citotóxico importante in vitro hasta 300 µg/ml, pero sí inhibió el crecimiento de tumor primario y de metástasis in vivo. UT inhibió la producción de TNF-α, IL-6 y ON in vitro. La actividad de NF-κB también resultó inhibida. Nuestros estudios muestran que UT contiene compuestos que ameritan ser más estudiados por sus efectos sobre procesos comunes a inflamación y cáncer.

**Palabras clave:** Uncaria tomentosa, cáncer, NF-κB, inflamación, B16/BL6 mouse

INTRODUCTION

*Uncaria tomentosa* is a species of vine from the Rubiaceae family, widely distributed throughout South and Central America, which has a long tradition of use as a folk medicine for the treatment of a variety of conditions including inflammation, cancer and gastrointestinal disorders (Reinhard, 1999). Previous studies have shown anti-inflammatory activity both in vitro (Sandoval-Chacón et al., 1998) and in mouse models of inflammation (Aquino et al., 1991; Caballero et al., 2005), and growth inhibitory effects in both tumor (Sheng et al., 1998) and normal cells (Akesson et al., 2003) in vitro. The role of chronic inflammation in tumor initiation and growth is well established (Coussens and Werb, 2002) so a dual action against both inflammation and cancer is not surprising. Studies have shown that anti-inflammatory drugs may be effective in cancer therapy and/or prevention (Thun et al., 2002), and the possible mechanisms of action of anti-inflammatory phytochemicals have been reviewed (Surh et al., 2001). *U. tomentosa* extracts have been shown to inhibit NF-κB (Sandoval-Chacón et al., 1998; Akesson et al., 2003), a transcription factor which represents an important link between chronic inflammation and cancer (Li et al., 2005) and which has been suggested as a possible target for the therapy of both (Bremner and Heinrich, 2002).

We have previously shown that intraperitoneal (i.p.) injection of an aqueous extract of *U. tomentosa*...
inhibited the TNF-α and IL-6 response to lipopolysaccharide (LPS) challenge, and the growth of both primary tumors and metastasis in mice (Caballero et al., 2005). In the present study, we report the in vivo anti-inflammatory and antitumor activities of a hydroethanolic extract, as well as an inhibitory effect on NF-κB.

MATERIALS AND METHODS

Plant material.

The bark of Uncaria tomentosa (Cat’s claw, Uña de gato), was obtained from the Peruvian Amazon region and identified by us. Aliquots were ground then macerated in a 70% ethanol in water solution for 21 days in the dark at room temperature. The suspension was then filtered under sterile conditions using Whatman No. 1 filter paper then adjusted to a stock concentration of 5 mg/ml, which was calculated from the dry weight of a lyophilized sample. This extract shall be termed UT.

Cells and animals.

The cell lines, B16/BL6 (murine melanoma), K1735 (amelanotic murine melanoma), HT29 (human colon carcinoma), A549 (human lung carcinoma), WEHI 164 (mouse fibrosarcoma), LEC (mouse liver endothelial cell line) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS - Gibco, BRL, USA), penicillin (100 Units/ml), streptomycin (100 μg/ml) and containing in addition glucose 0.45% (HT29 cells), and L-glutamine 2 mM (A549 cells). Human peripheral blood monocytes (huPBMC) were obtained from healthy donors by standard Ficoll/Hypaque gradient centrifugation and cultured in RPMI-1640 10% FBS. Chopped spleens from C57BL/6 mice were ground through a wire mesh screen. After removal of detritus and lysis of red blood cells with 0.085% sodium citrate, adherent cells we re removed by overnight incubation in plastic culture flasks. The non-adherent cells (muSplen) were harvested, counted and cultured in RPMI-1640 10% FBS. Murine peritoneal macrophages (muPM) were collected from C57BL/6 mice 4 days after a peritoneal injection of 2 ml of 4% thioglycollate. The cells were washed, seeded into culture flasks in RPMI-1640 10% FBS, and non-adherent cells discarded after 3 h. The adherent cells were then used immediately.

Female C57BL/6 mice (7–9 weeks old) were obtained from the Animal Facility, IVIC and fed with standard pellet diet and water ad libitum. All animal experiments were performed according to internationally accepted guidelines for the treatment of animals in research.

Cytotoxicity.

Cells were plated at 2.5 - 5 x 10^4 cells / well in 100 μl of culture medium in flat-bottomed 96 well plates and allowed to attach for 24 h. Different concentrations of UT in 100 μl culture medium were then added. Control wells were set up containing equivalent quantities of ethanol, which in no case exceeded 1%. No effect was observed due to the ethanol. After a further 24 h, the number of viable cells was assessed using the MTS/PMS chromogenic assay (Promega Corp., USA) according to the manufacturer’s instructions.

Inflammatory response in vitro.

Peritoneal macrophages were activated with 10 μg/ml lipopolysaccharide (LPS - E. coli serotype 055:B5, Sigma, USA) for 24 h in the presence of UT, and then the concentrations of TNF- α, IL-6 and nitric oxide (NO) were measured in the supernatants. TNF- α was quantified using the WEHI 164 cell bioassay (Espevik and Nissen-Meyer, 1986), IL-6 with a commercial ELISA assay (R & D Systems Inc., MN, USA) and NO using the Griess reaction (Sandoval-Chacón et al., 1998).

Inflammatory response in vivo.

Mice were injected intraperitoneally (i.p.) with different doses of LPS in 100 μl of PBS. After 1 h, blood was collected by heart puncture under ether anesthesia. Serum was separated and assayed for the two cytokines and NO as described above. In order to evaluate the effect of UT on the inflammatory response, mice were injected i.p. with 50 μg UT on 3 consecutive days prior to LPS challenge.

Lung metastasis.

At day 0, mice were inoculated in the lateral tail vein (i.v.) with 10^5 B16/BL6 cells in 100 μl PBS. Two treatment protocols with UT were performed a) intraperitoneal (i.p.) injection of 50 μg of UT in 100 μl PBS / 25% ethanol on days -2, -1 and 0, and b) i.p. injection of the same dose of extract 5 times per week starting from on day 0 up to day 21. Control
animals received 100 μl PBS / 25% ethanol. On day 23, the animals were sacrificed with ether; the lungs were removed, placed for 5 min in 3% H₂O₂ in H₂O and fixed in Bouin's solution. The purpose of the H₂O₂ was twofold: to bleach hemorrhages which could be mistaken for metastases, and to inflate the lungs, facilitating the evaluation of metastases under the dissecting microscope. Animals were challenged with LPS prior to sacrifice, in order to measure serum TNF-α and IL-6 levels as described above.

Primary tumors.

Primary tumors were induced by the subcutaneous (s.c.) injection of 5 x 10⁴ B16/BL6 cells in 100 μl PBS into the hind limb. The mice were injected i.p. with 50 μg of UT 5 times per week starting from on day 0 up to day 21. Tumor size was measured in two dimensions with a vernier gauge. Animals were challenged with LPS prior to sacrifice, in order to measure serum TNF-α and IL-6 levels as described above.

NF-κB luciferase assay.

HeLa cells were transiently transfected with the NF-κB luciferase reporter system (Stratagene, La Jolla, CA, USA) according the manufacturer’s instructions. Transfected cells were seeded into 96-well plates, allowed to adhere overnight then treated for 1 h with different concentrations of UT. The cells were then stimulated for a further 4 h with 25 ng/ml huTNF-α (BD Biosciences, Palo Alto, CA, USA). Luciferase activity was measured using the Steady-Glo assay kit (Promega, Madison, WI, USA), in a 96-well luminometer. Dexamethasone and paclitaxel, which are known to inhibit and stimulate NF-κB activity respectively, were included as controls.

Statistical analysis.

Each experiment was performed at least three times and results are expressed as the mean ± standard deviation. The unpaired Student’s t test with the Welch correction was used to assess the statistical significance of the differences. A confidence level of P < 0.05 was considered significant.

RESULTS

Cytotoxicity.

Initial in vitro experiments (Fig. 1) showed an inhibitory effect of UT on 24 h cell growth only at the higher concentrations of 100 and 300 μg/ml. Inhibition was less than 50% in all cases. Three tumor cell lines (HT-29, K1735 and WEHI) and the three primary cell preparations (huPBMC, muSplen and muPM) were more sensitive to the extract than the B16/BL6, A549 and LEC cells.

Effect of UT on the inflammatory response in vitro and in vivo.

The TNF-α response of mouse peritoneal macrophages to LPS was reduced by 48% in the presence of 100 μg/ml UT (Fig. 2), although this reduction was not significant due to variability in the results. The IL-6 and NO responses were significantly reduced by 35% and 62% respectively (P < 0.001). This result was not due to a direct cytotoxic effect, as no change in the viability of these activated cells was observed at this concentration of UT (results not shown). A similar reduction in the inflammatory response was observed in vivo, when mice were injected i.p. with 50 μg UT on 3 consecutive days prior to challenge with different doses of LPS (Fig. 3). The TNF, IL-6 and NO responses were reduced by 63, 59 and 57% respectively when the animals treated with UT were challenged with the highest dose of LPS. Again, only the results for IL-6 and NO were significant due to variability in the TNF response to LPS.
**Figure 2.** Inhibition by UT of the inflammatory response of mouse peritoneal macrophages to LPS. Cells were activated with 10 μg/ml LPS for 24 h in the presence of 100 μg/ml UT extract. TNF-α, IL-6 and NO levels were then measured in the supernatants. (mean ± S.E.M., n = 3). **P < 0.001**

**Inhibition by UT of primary tumor growth and metastasis in mice.**

Mice were inoculated s.c. with B16/BL6 cells and the effect of i.p. UT on primary tumor growth was measured. At all time points after the appearance of the tumor, there was a very significant inhibition of tumor growth in the animals treated with UT (Fig. 4A). This effect was most notable at earlier times with the treated tumors measuring approximately 75% less than the controls up to day 16, but only 50% less at day 22. Effect of UT on the inflammatory response to LPS in tumor-bearing animals.

As basal levels of serum TNF-α and IL-6 are very low in animals with either primary tumors or metastases, we evaluated the effect of UT on the inflammatory response to a low dose of LPS (3 μg / animal) in tumor-bearing animals, prior to sacrifice.

**Figure 3.** Inhibition by UT of the inflammatory response to LPS in mice. Animals were pretreated with 50 μg/ml UT i.p. on 3 days, then challenged with different doses of LPS. After 1 h, blood was extracted and the serum assayed for TNF-α, IL-6 and NO. (mean ± S.E.M., n = 3). * P < 0.05.

Figure 5 shows that both the TNF-α and IL-6 responses to low dose LPS were much greater in animals with primary tumors than in animals without tumors (compare with Fig. 3). In contrast, very little priming of the inflammatory response to low dose LPS was observed in the animals with metastasis. The TNF-α and IL-6 responses in animals with primary tumors were greatly inhibited by UT treatment, (85% and 81% respectively). Although TNF-α levels were lower in the untreated animals with metastasis, an important inhibitory effect (81%) was observed after UT treatment. The already almost basal levels of IL-6 in these animals were unaffected by UT.
Figure 4. Effect of UT treatment on primary tumor growth and metastasis in mice. A. C57Bl/6 mice were inoculated s.c. with B16/BL6 tumor cells to initiate a primary tumor and injected i.p. 5 times a week up to day 21 with 50 μg UT. Tumor growth was assessed with a vernier gauge. B. Mice were inoculated i.v. with B16/BL6 cells. Treatment with UT consisted of 50 μg i.p. and the 3 days prior to inoculation. Lung metastases were counted on day 22. C. Mice were inoculated i.v. with tumor cells, as in B., but treated with UT post-inoculation as in A. (mean ± S.E.M., n = 10). * P < 0.005, ** P < 0.0005.

Figure 5. Effect of UT on the inflammatory response to LPS in tumor-bearing mice. C57Bl/6 mice were inoculated s.c. or i.v. with B16/BL6 tumor cells to produce primary tumors or metastases, respectively, then injected i.p. 5 times a week up to day 21 with 50 μg UT (corresponding to Fig 4A and 4C). One h before sacrifice, the animals were challenged with 3 μg LPS. Blood was extracted and the serum assayed for TNF-α and IL-6. (mean ± S.E.M., n = 3). * P < 0.05.

Effect of UT on the NF-κB response to activation by TNF-α in HeLa cells.

The effect of UT on the NF-κB response of HeLa cells to TNF-α was determined in a luciferase reporter assay. UT at 10 μg/ml slightly stimulated NF-κB activity, but was inhibitory (47%) at 100 μg/ml (Fig. 6). The highest dose of dexamethasone used (30 μM), a known NF-κB inhibitor, inhibited the response by 38%. A change of medium after a preincubation with UT only slightly reduced the degree of inhibition, suggesting that the effect was not due to a direct interaction between the crude extract and LPS or TNF-α (results not shown). Preliminary experiments also confirmed that UT was not cytotoxic for these cells at the doses used in these short term assays (results not shown).
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Figure 6. Effect of UT treatment on NF-κB activity. HeLa cells transfected with the NF-κB/luciferase reporter plasmid were treated for 1 h with different concentrations of UT, then stimulated for a further 4 h with 25 ng/ml huTNF-α. DEX – dexamethasone (30 μM), PAC – paclitaxel (1 μM) – see Materials and Methods. (mean ± S.E.M., n = 3).

DISCUSSION

Our previous studies indicated that an aqueous extract of UT was not cytotoxic for a range of cells at concentrations up to 3 mg/ml (Caballero et al., 2005). Here we show that the hydroethanolic extract of UT, was more cytotoxic for some of the tumor cell lines and the primary cells, but not for the B16/BL6 melanoma cells. We were unable to test concentrations higher than 300 μg/ml, as a precipitate began to appear in the cultures at 1 mg/ml. A review of several reports suggests that extracts of U. tomentosa may show some antiproliferative activity (Sandoval-Chacón et al., 1998; Sheng et al., 1998; Riva et al., 2001; Akesson et al., 2003; De Martino et al., 2006) although the effects were observed in the “mg/ml” range. Santa María showed that aqueous extracts of U. tomentosa were not toxic for Chinese hamster ovary cells (CHO) in 3 different bioassay systems (Santa Maria et al., 1997). In contrast, U. tomentosa was reported to attenuate peroxynitrite-induced apoptosis in HT29 and RAW 264.7 cells (Sandoval-Chacón et al., 1998). Considering these results and the dose of UT we used in the in vivo experiments (50 μg UT/day), it is difficult to conclude that the inhibitory effect seen with the primary tumors and metastasis was due to a direct effect on tumor cell proliferation or viability.

Our results confirm previous reports of the anti-inflammatory action of extracts of Cat’s Claw (Aquino et al., 1991). Although it has been reported that an aqueous extract of this plant inhibited TNF-α and nitrite production by the RAW 264.7 mouse macrophage cell line in vitro (Sandoval et al., 2000; Sandoval et al., 2002), as far as we are aware this is the first report of its inhibitory effect on inflammatory cytokine production in vivo while there have been two reports of a stimulatory effect of Uncaria extracts on IL-6 production (Lemaire et al., 1999; Eberlin et al., 2005). Evidently such results must be interpreted taking into account the nature of the preparation used, and its route of administration. Aguilar reported that a hydroethanolic extract was more active than a water extract in the mouse paw edema assay (Aguilar et al., 2002). Such extracts may be expected to contain compounds with a wider range of polarities, including bioactive alkaloids, than a water extract but also less desirable compounds such as tannins (Sheng et al., 2005; Pilarski et al., 2006).

Our finding that UT inhibits the inflammatory response as well as tumor growth and metastasis, in the same mouse model is interesting considering the proven relationship between inflammation and cancer. UT may contain compounds which separately exert anti-inflammatory or anticancer effects through different mechanisms, or the results may be due to a compound, or group of compounds, acting through a common mechanism. Plant components with both activities including flavonoids and terpenes have been reported (Evans and Taylor, 1983; Middleton et al., 2000). In a previous study, we showed that blocking TNF-α with a TNF receptor construct decreased serum TNF-α and IL-6 levels after LPS challenge in tumor-inoculated mice (Cubillos et al., 1997) as well as reducing the number of lung metastases in the same animals.

Previous studies have shown that aqueous extracts of U. tomentosa inhibit NF-κB activity (Sandoval-Chacón et al., 1998; Akesson et al., 2003), whereas Aguilar reported greater inhibitory activity in a hydroethanolic extract (Aguilar et al., 2002). Here, we confirm that our hydroethanolic extract was also inhibitory for NF-κB, at concentrations that were not cytotoxic. However, NF-κB is an anti-apoptotic factor in cells and its constitutive expression may be important in the survival of tumor cells (Aggarwal, 2004). Thus, the anticancer effects of anti- NF-κB compounds derived from plants may derive from an inhibition of inflammatory cells, as well as a direct effect, at higher concentrations, on the tumor cells themselves.
The biomedical applications of plants with anti-inflammatory activity are becoming increasingly important given the links which have been established over the last decade or so between cancer and inflammation. This field becomes increasingly interestingly since it has been found that many antitumor drugs, such as paclitaxel activate NF-κB, thus counteracting their own cytotoxicity (Karin et al., 2002). Anti-inflammatory drugs that act through NF-κB, whether derived from plants or not, may be potential sensitizers to enhance the effectiveness of conventional cancer chemotherapy.

CONCLUSION

The results suggest that the anticancer activity of this hydroethanolic extract of *U. tomentosa* in this model may, to a large extent, be due to its anti-inflammatory properties rather than to a direct cytotoxic effect.

REFERENCES


