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IN VITRO AND *IN VIVO* ANTI-TUMORAL EFFECT OF CURCUMIN AGAINST MELANOMA CELLS

Johann ODOT[1], Philippe ALBERT[1]*, Annie CARLIER[1], Michel TARPIN[1], Jérôme DEVY[2] and Claudie MADOULET[2]

[1]*Laboratoire de Biologie Cellulaire et Moléculaire, EA 3306 U.F.R Sciences, Reims, France*
[2]*Laboratoire de Biochimie et Biologie Moléculaire, EA 3306, U.F.R. Pharmacie, IFR 53 Biomolécules, Reims, France*

Curcumin, the active ingredient from the spice turmeric (*Curcuma longa* Linn), is known to be an anti-oxidant and an anti-inflammatory agent. It has been demonstrated recently to possess anti-angiogenic effects and pro-apoptotic activities against Ehrlich ascites tumor cells. In the current study, curcumin was found to be cytotoxic *in vitro* for B16-R melanoma cells resistant to doxorubicin either cultivated as monolayers or grown in three-dimensional (3-D) cultures (spheroids). We have demonstrated that the cytotoxic effect observed in the 2 culture types can be related to the induction of programmed cell death. In our *in vivo* studies, we examined the effectiveness of a prophylactic immune preparation of soluble proteins from B16-R cells, or a treatment with curcumin as soon as tumoral appearance, alone or in combination, on the murine melanoma B16-R. The combination treatment resulted in substantial inhibition of growth of B16-R melanoma, whereas each treatment by itself showed little effect. Moreover, animals receiving the combination therapy exhibited an enhancement of their humoral anti-soluble B16-R protein immune response and a significant increase in their median survival time (>82.8% *vs.* 48.6% and 45.7% respectively for the immunized group and the curcumin-treated group). Our study shows that curcumin may provide a valuable tool for the development of a therapeutic combination against the melanoma.

Key words: *curcumin; apoptosis; immune preparation; melanoma*

Resistance to chemotherapy is a major problem in oncology. Multiple mechanisms of intrinsic multidrug resistance (MDR) were identified using *in vitro* cell lines. These mechanisms involved drug efflux but also intracellular drug distribution, detoxification, DNA synthesis and repair.[1–4] The fact that tumor cells use multiple mechanisms to escape from chemotherapeutic drugs has stimulated interest in developing immunotherapeutic strategies that target both humoral and cellular immunity to malignant cells. The use of modified tumor cells,[5–9] whole tumor lysates,[10] tumor lysate-pulsed dendritic cells,[11,12] nucleic acid[13–15] or proteins[16–18] as vaccines to induce or increase antitumoral immunity has been explored over the last 30 years. Melanoma is the main cause of death in patients with skin cancer. Of all skin disorders, malignant melanoma has the highest mortality rate (10–15%), and accounts for 1–2% of all cancer-related deaths among Caucasians. In view of the aggressive growth of invasive malignant melanoma, early diagnosis and treatment are very important.

Turmeric powder, the powdered dry rhizome of *Curcuma long* Linn, gives specific flavor and color to curry[19] and has been traditionally used as a folk medicine to treat inflammatory disorders.[20] The active principle in turmeric powder, curcumin, was discovered to be an antioxidant[21] and an angiogenesis inhibitor.[22] Moreover, curcumin inhibits several signal transduction pathways including those involving protein kinase-C[23] and the transcription factor NF-κB.[24] Curcumin has chemopreventive activity and inhibits tumor initiation by some carcinogens[25,26] and tumor promotion induced by phorbol esters.[27] Curcumin has entered into Phase I clinical trials for chemoprevention by the National Cancer Institute[28] and it has been shown recently that it induces apoptosis in Ehrlich ascites carcinoma cells by upregulation of Bax, release of cytochrome *c*, and activation of caspase-3.[29] Studies on this biologically active natural compound have to be developed further and translated into therapeutic treatment of cancers where formation of metastases are a major cause of morbidity and mortality.

We investigate the capacity of curcumin to elicit anti-tumor activity both *in vitro* and *in vivo* on B16 resistant to doxorubicin mouse melanoma cells (B16-R). Moreover, as it has been demonstrated that curcumin activated Ehrlich's ascites carcinoma cell killing and restored tumor induced immuno depletion of the host,[29] we test whether this polyphenolic compound can enhance the potentiality of a simple immune preparation against the poorly immunogenic B16-R melanoma. We show that curcumin induces programmed cell death in B16-R cells and optimum *in vivo* anti-melanoma curative effect is obtained in combination with a prophylactic immune preparation using soluble proteins extracted from B16-R cells.

MATERIAL AND METHODS

Animals

Female B6D2F1 mice (6–8 weeks old) were purchased from the Charles River Laboratories (Iffa Credo, L'Arbresle, France) and housed at the animal maintenance facility of the Centre de Biotechnologies, U.F.R. Sciences, Reims, France.

Cells and culture conditions

A murine melanoma cell subline, denoted B16-R, resistant to 3.5×10^{-7} M doxorubicin, derived from the ATCC stock and isolated at the Tumor National Institut in Milan,[30] was isolated by stepwise selection in increasing concentrations of doxorubicin. B16-R cells were grown in a 5% CO_2 atmosphere at 37°C, in RPMI 1640 medium (Invitrogen, Cergy-Pontoise, France) supplemented with 10% heat-inactivated FBS (Invitrogen). To generate spheroids, exponentially growing monolayer B16-R cells were detached by trypsinization and 1 ml culture medium containing 1×10^5 cells was added to each well of a 24-well microplate, previously coated with 400 μl 1.33% agarose Type II (Sigma, St. Quentin-Fallavier, France). Plates were incubated 24 hr on a rocker designed for three dimensional (3-D) agitation (70 cycles/min) at 37°C under 5% CO_2 and humidified atmosphere.

Assay for cytotoxicity of curcumin

A colorimetric assay utilizing the tetrazolium salt: MTT (Sigma) was used. Briefly, 1×10^4 B16-R cells were cultivated as monolayer culture for 12 hr. They were then incubated in 200 μL of RPMI, 10% FBS containing curcumin (Sigma) at final concentrations from 1–100 μM in 96-multiwell plates for 24–48 hr. After these incubations, cells were washed twice in PBS and 500 μl of fresh culture medium containing MTT (0.3 mg/mL) were added

*Correspondence to: Laboratoire de Biologie Cellulaire et Moléculaire, UFR des Sciences, BP 1039, 51687 Reims Cedex 2, France.
Fax: +33-03-26-91-32-82. E-mail: philippe.albert@univ-reims.fr

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for colorimetric assay. Cell survival assay using curcumin was also studied on B16-R cells cultivated as spheroids. Spheroids (3 days old) were incubated in 1 mL of RPMI-FBS containing curcumin at final concentrations from 1–200 μM, in 24-multiwell plates for 24–48 hr. Spheroids were then mechanically dissociated by pipetting, washed twice with PBS and 500 μL of fresh culture medium containing MTT (0.3 mg/mL) were added. In the 2 cases, after 3 hr incubation at 37°C with MTT, DMSO was added to solubilize crystals (200 μL for monolayer cultures and 750 μL for spheroids). Cell viability was determined by measuring, optical density differences between 550–650 nm using a model 550 microplate reader (Bio-Rad, Marnes la Coquette, France). The surviving fraction of cells was determined by dividing the mean absorbance values of treated samples by the mean absorbance of untreated control samples.

Genomic DNA fragmentation analysis

B16-R cells cultured as monolayers with or without curcumin were treated for genomic DNA isolation[31] and analyzed in 1.8% agarose gel to visualize the specific DNA ladder observed during programmed cell death. At selected time points, spheroids of B16-R cells treated with or without curcumin were fixed with 10% formalin in PBS for 4 hr at 4°C. The samples were then dehydrated with increasing ethanol concentrations, cleared with clearene (Surgipath Medical Industry, Richmond, IL) and embedded in paraffin. Sections of 5μm were cut, mounted on slides and rehydrated with clearene and decreasing concentrations of ethanol and water. The cell death identification was carried out by TUNEL assay, using the Apoptag *In Situ* Apoptosis Detection Kits (Appligene-Oncor, Illkirch, France) according to the protocol providing. Fragmented DNA, generally observed during cell death, was labeled using the method of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL). Preparations were incubated with 20 μg/ml proteinase K for 15 min at room temperature for exposing the DNA. After washing in PBS, the slides were covered with TdT (0.3 U/μL; Intergen, NY) and digoxigenin-dUTP (Intergen) in TdT buffer (30 mM Tris, pH 7.2, 140 mM Na-cacodylate, 4 mM $MgCl_2$, 0.1 mM DTT) and incubated for 75 min at 37°C in a humidified chamber. The reaction was stopped by transferring the slides in 300 mM NaCl and 30 mM Na citrate buffer. Detection of digoxigenin-dUTP was carried out using sheep polyclonal anti-digoxigenin horseradish peroxidase-conjugated antibodies (1:200) for 45 min at room temperature. Each section was then reacted with 3,3′-diaminobenzidine (DAB) solution containing 0.05% DAB and 0.03% H_2O_2 in PBS for 5–10 min. The cells were then counterstained with 0.5% (w/v) methyl green and dehydrated in *N*-butanol and clearene. Negative controls were incubated without enzyme but with antibodies.

Immune preparation

Viable tumor cells were suspended at 2×10^7 cells per mL in NaCl (0.85%). After 4 cycles of rapid freeze (−160°C) and thaw (37°C), the tumor cell suspension was then centrifuged (14,000*g*/15min/4°C) and the supernatant collected. The amount of B16-R soluble proteins in the supernatant was estimated using the BC Assay Kit (Uptima, Montluçon, France) according to manufacturer's instructions. The final protein concentration was 800 μg/mL in NaCl (0.85%).

Tumor induction and in vivo *treatments*

For tumor induction, all mice were shaved on the right flank and challenged subcutaneously (s.c.) with 1×10^6 viable B16-R cells in PBS. For the prophylactic treatment, the immune preparation was administrated on the left flank weekly for 4 weeks before tumor cell challenge. For each injection, each mouse received 40 μg of soluble B16-R proteins in saline solution (0.85% NaCl) in the presence of complete Freund's adjuvant (Interchim, Montluçon, France) for the first injection and then incomplete Freund's adjuvant for the subsequent injections. For *in vivo* curcumin treatment, mice were daily injected intraperitoneally (i.p.) with 25 mg/kg body weight (bw) (100 μL/mouse) after tumor appearance. The curcumin was dissolved in DMSO to a final concentration of 500 mM and further dilutions were done in NaCl (0.85%). The size of the tumors was assessed in a blinded, coded fashion twice weekly and recorded as tumor area (cM^2) by measuring the largest perpendicular diameters with calipers.

In vivo *survival assay*

B16-R melanoma cells (1×10^6) were s.c. injected into B6D2F1 mice. Treated mice were daily injected either with 25 mg/kg bw curcumin after tumoral appearance or received only B16-R soluble proteins s.c. before tumor challenge. In another group, mice were injected with B16-R soluble proteins before tumor challenge prior they were daily treated with curcumin (25 mg/kg bw) after tumor development. The life spans of the animals in each group were estimated. The percentage increase in median survival time (% IMST) was calculated from the formula: % IMST = $[(T - C)/C] \times 100$, where T is the number of days treated animals median survived and C is the number of days control animals median survived. The experiment was repeated twice.

RESULTS

Effects of curcumin on B16-R cells grown as monolayers

We first investigated the effect of curcumin on B16-R melanoma cells in monolayer cultures. Results are shown in Figure 1. The effect of curcumin on the B16-R cell viability was observed for low drug concentrations and lessened the cell viability in a dose-dependent manner showing the optimum activity with 50 μM regardless of the incubation time. For 24- and 36-hr incubation with curcumin, the IC_{50}-values were about 27 μM. A 48-hr incubation with curcumin slightly reduced IC_{50}-value from 27 μM to 18 μM. In all cases curcumin is cytotoxic for B16-R melanoma cells.

Effect of curcumin on B16-R spheroid growth

The effect of curcumin was studied on B16-R cells grown in 3-D cultures (called spheroids) that are more *in vivo*-like solid tumors. As shown in Figure 2, spheroids of B16-R cells are more resistant to the lethal effect of curcumin than monolayer cells (see drug concentrations <100 μM). In our conditions for B16-R spheroids, the IC_{50}-values were 160 μM for 48 hr and 175 μM of curcumin after 24/36 hours incubation with the drug.

Effect of curcumin on apoptosis of B16-R cells in monolayers

As it has been reported previously that curcumin induced apoptosis in several cell types.[32] We have studied whether the lethal effect of curcumin observed on B16-R melanoma cells was due to induction of programmed cell death. One of the features distinguishing apoptosis from necrosis is the early onset of specific endonuclease-mediated cleavage of cellular DNA into nucleosome ladders. We examined whether curcumin can elicit a similar pattern of DNA fragmentation in curcumin-treated B16-R monolayer cultures (Fig. 3). DNA analysis of cells exposed to 100 μM curcumin for 24 hr showed the typical apoptosis DNA cleavage (Fig. 3*a*, *lane 2*) whereas no such pattern was seen in untreated cells (Fig. 3*a*, *lane 1*). No similar DNA fragmentation was observed in cells treated with 200 μM curcumin for 24 hr. The DNA fragments were approximately 180 base pairs (bp), suggesting that all DNA was cleaved in fragments that were associated with mononucleosomes. At concentration <100 μM, no DNA fragmentation was observed (data not shown). This result was not surprising because a simple 1/10 dilution from sample showing a DNA ladder after agarose gel electrophoresis did not exhibit the characteristic apoptotic DNA pattern. It can be concluded that for concentrations between 100–200 μM, curcumin induced B16-R cell killing *via* programmed cell death in a dose-dependent manner. Moreover, we observed that the apoptotic effect of curcumin on B16-R cells was time dependent because when the cells were exposed to 100 μM curcumin for 48 hr instead 24 hr, almost the DNA fragments were 180 bp (Fig. 3*b*, *lane 2*), whereas a DNA

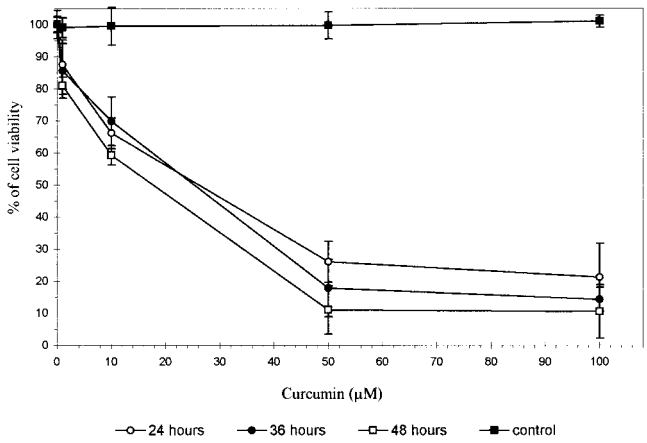

FIGURE 1 – Effect of curcumin on B16-R cells. Cells (1×10^4) were cultivated as monolayer for 12 hr before incubation with curcumin (1–100 μM) at different times. IC_{50} were determined by MTT assay. The data represented means ± SEM of 6 independent experiments.

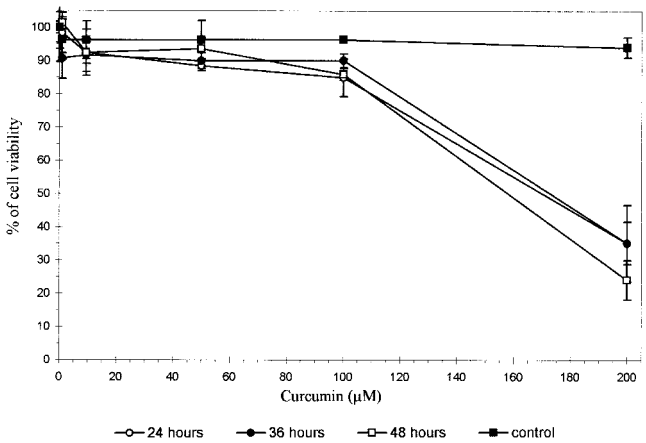

FIGURE 2 – Effect of curcumin on spheroids of B16-R. Three-day-old spheroids were incubated with curcumin (1–200 μM) during various incubation times. IC_{50} were determined by MTT assay. Data were representative of means ± SEM of 4 independent experiments.

ladder was seen at the same curcumin concentration after 24 hr of treatment (Fig. 3*a*, *lane 2*). In agreement with this result, a typical apoptotic DNA ladder was observed when B16-R cells were exposed to 10 μM curcumin for 48 hr. No DNA fragmentation was seen after only 24 hr of treatment (data not shown).

Effect of curcumin on apoptosis of B16-R spheroids

We have demonstrated that the cytotoxic doses of curcumin were different on B16-R cells grown as spheroid or monolayer cultures (Figs. 1,2). We then examined whether this treatment also induced apoptosis of B16-R cells cultivated in 3-D cultures. In spheroid, cells located at the periphery are alive and actively dividing, deeper cells are non-cycling. In the aggregate center, cells are dead and form a necrotic core. The size of the necrotic center is variable and according to this particular morphology, it is quite impossible to examine DNA ladder formation in cells from the viable rim by agarose gel electrophoresis. We examined the possibility of apoptosis in such cells growing in spheroids after curcumin treatment by means of TUNEL staining (Fig. 4). When spheroids were grown in presence of 100 μM curcumin for 48 hr (Fig. 4*a*), TUNEL-positive cells were observed in the necrotic center (that is quite normal) and the outer 3–5 cell layers (about 75 μm). Deeper cells above the necrotic center that are reproductively viable and quiescent cells located more centrally were TUNEL-negative. This is probably due to the limited penetration of curcumin at 100 μM. The small amount of TUNEL-positive cells observed in Figure 4*a* is in agreement with our previous results shown in Figure 2 for the same concentration of curcumin and 48 hr treatment where about 85% of cells were viable. To illustrate the limited penetration of curcumin and its apoptotic effect, we incubated the spheroids in 200 μM curcumin instead 100 μM for the same time (48 hr) (Fig. 4*b*). Contrary to the previous result (Fig. 4*a*), all cells of the peripheral layers (about 300 μm) were TUNEL-positive. This indicates that the apoptotic effect of curcumin on B16-R cells grown as spheroid is quite achieved and agrees with Figure 2 where the surviving fraction of B16-R cells in spheroids was between 17–29%. We can conclude that curcumin is capable of inducing apoptosis in B16-R melanoma cells cultivated as monolayers or grown in three-dimensional cultures (spheroids).

According to our *in vitro* results on the growth of B16-R cells and spheroids, it has been demonstrated that curcumin has anti-cancer effects on colon-tumor promotion[33] and is an angio inhibitory compound. We have investigated the capacity of curcumin to elicit anti-tumor activity *in vivo* against the poorly immunogenic and highly metastatic chemoresistant B16 melanoma cells.

In vivo *studies of curcumin on tumor development*

Figure 5 shows the delay and percentage of tumoral appearance in 3 groups of animals. All mice were s.c. challenged with 1×10^6 B16-R cells. In the control group during 7 days post-challenge, no tumor can be detected on the right flank of mice, and 100% of mice developed a melanoma. In the group treated with curcumin, no difference was observed with the control group. This result is quite normal because we have chosen to study the therapeutic effects of curcumin and not their prophylactic counterparts. To test whether curcumin used in therapy could enhance a potential simple immune preparation against this poorly immunogenic tumor based on the prophylactic injection of soluble B16-R proteins, a third group was treated first, with 4 consecutive injections (in a 7-day time frame as indicated in Material and Methods) of soluble B16-R proteins before tumor challenge. The mice from this group were then treated with curcumin as soon as tumoral appearance. As observed in Figure 5, tumors were always detectable within 2–6 days later than in the previous groups indicating a very slight effect

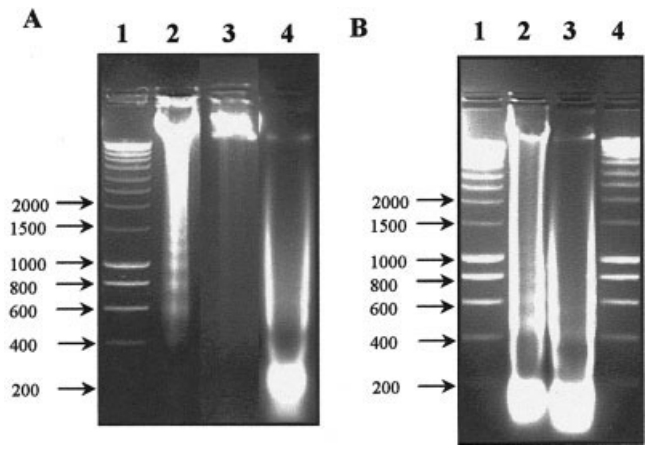

FIGURE 3 – DNA analysis of curcumin-treated B16-R cells. B16-R cells (2×10^6) were cultivated for 12 hr before curcumin treatment. DNA from B16-R cells (20 μg/well) was analyzed on 1.8% agarose gel-electrophoresis. (*a*) DNA molecular weight marker (*lane 1*) is indicated in base pairs (bp) and DNA from control cells (*lane 3*). B16-R cells were treated with curcumin 100 μM (*lane 2*) and 200 μM (*lane 4*) for 24 hr. (*b*) DNA molecular weight marker (*lanes 1,4*). DNA from B16-R cells treated 48 hr with 100 μM (*lane 2*) and 200 μM (*lane 3*) of curcumin.

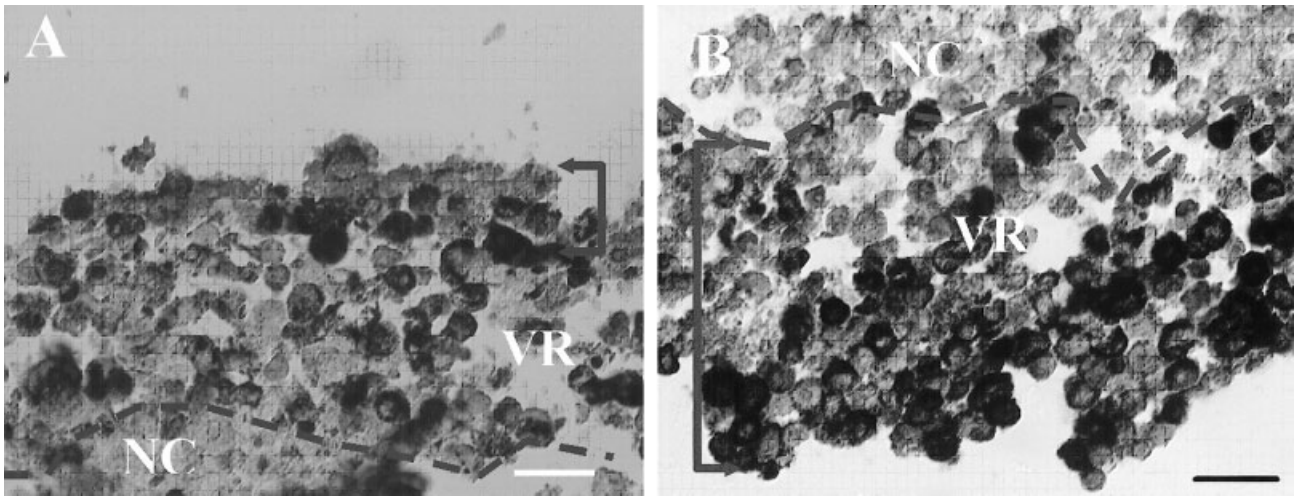

FIGURE 4 – *In situ* analysis of DNA fragmentation by TUNEL assay on spheroids of B16-R cells treated with curcumin. Three-day-old spheroids cultivated 48 hr with 100 μM (*a*) or 200 μM (*b*) of curcumin. (*a*) Only the circumference of the viable rim (roughly 2 layers of peripheral cells, red bracket) shows TUNEL-positive cells (brown color). (*b*) All the cells of the viable rim located above the necrotic center are TUNEL-positive (red bracket). The black pigment corresponds to melanin. The necrotic center is always TUNEL-positive (very light brown). The sections were counterstained with methyl green. VR, viable rim; NC, necrotic center. Scale bars = 100 μm.

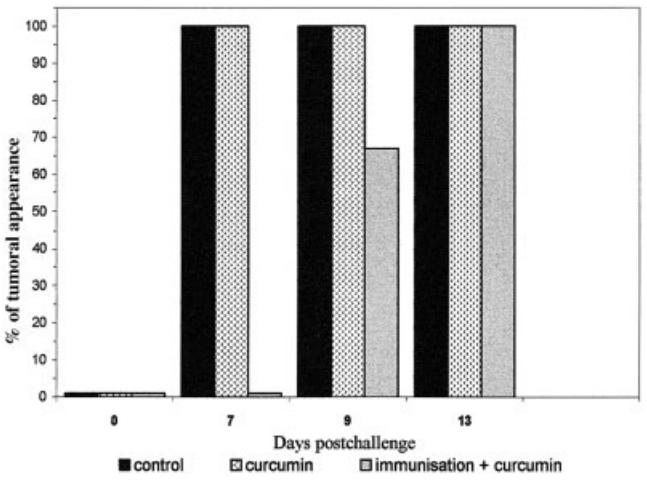

FIGURE 5 – Tumoral appearance in mice after B16-R injection. B6D2F1 female mice (6/group) were injected s.c. with 1×10^6 viable B16-R cells on the right flank. Control mice (filled black bars) received no treatment before and after tumor challenge. In the curcumin group (filled stippled bars) mice were injected i.p. each day with 25 mg/kg bw of drug after tumor development. In the immunized + curcumin group (filled grey bars), mice received 4 consecutive B16-R soluble protein injections (in a 7-day time frame as indicated in Material and Methods) before tumor challenge. They were then injected i.p. each day with 25 mg/kg bw curcumin after the tumor was developed.

of our immune preparation against the weakly immunogenic B16-R cells. Administration of such formulation delayed tumor growth slightly when initiated before B16-R cell injection but had no effect when immunization started with melanoma cells implantation (data not shown). It can also be observed in this mice group that the tumoral appearance was not homogeneous (between 9–13 days post-tumor challenge). The slight increase in the delay of tumoral appearance can be ascribed to the prophylactic treatment. When we analyzed the immune serum from mice that received soluble B16-R proteins before melanoma cell injection, a slight humoral immune response anti-B16-R proteins can be detected. No antibody was found in sera from control mice (data not shown). Despite this immune response, 100% of mice developed a tumor. In previous experiments, we found that immune preparation with soluble proteins recovered in supernatant of B16-R cells lysed was quite ineffective against B16-R melanoma and no tumor rejection was observed. We hypothesized that this might be due to insufficient presentation of tumor antigens by host APCs or low amounts of injected soluble B16-R immunogens.

Effects of curcumin associated with immunization of soluble proteins from supernatants of B16-R cells

We combined immunization with soluble proteins of B16-R cells and therapeutic treatment with curcumin when the tumor was developed. The tumor growth in animals treated daily with curcumin and their controls are shown in Figure 6. The combination of our immune preparation and curcumin treatment, (but not immunization or curcumin alone) resulted in substantial inhibition of growth of B16-R melanoma. By Day 40 after tumoral appearance, tumor size in the immunized + curcumin group averaged about 4.25 cM^2 compared to those of approximately 9 cM^2 in curcumin or immunized groups and 16 cM^2 in control mice. It can be concluded that the tumor growth in the combination therapy is about 4× lower than control mice and 2× lower than each treatment alone. It can also be observed that in the group receiving the combination treatment, the tumor growth is very heterogeneous (R^2 was ±0.38). No heterogeneity of tumor growth was seen in any of the groups without soluble B16-R protein immunization (control group and curcumin group, where R^2 was ≈1). When we analyzed the sera of animals receiving the combination treatment, more soluble B16-R proteins were detected in sera of animals receiving the combination treatment, than in sera of mice from the immunized group (data not shown). This result is consistent with an enhancement of the immune response in mice treated with curcumin. The inhibition of growth of B16-R melanoma observed in the immunization + curcumin group could be explained by a best humoral response anti-soluble B16-R protein The observation that the combination therapy had at least some effect in reducing tumor growth led us to test its effectiveness in increasing survival.

Effect of curcumin on survival time of mice

As shown in Figure 7, mice challenged with 1×10^6 viable B16-R cells, without any treatment succumbed by Day 48 after injection. Curcumin by itself prolonged survival as did immuni-

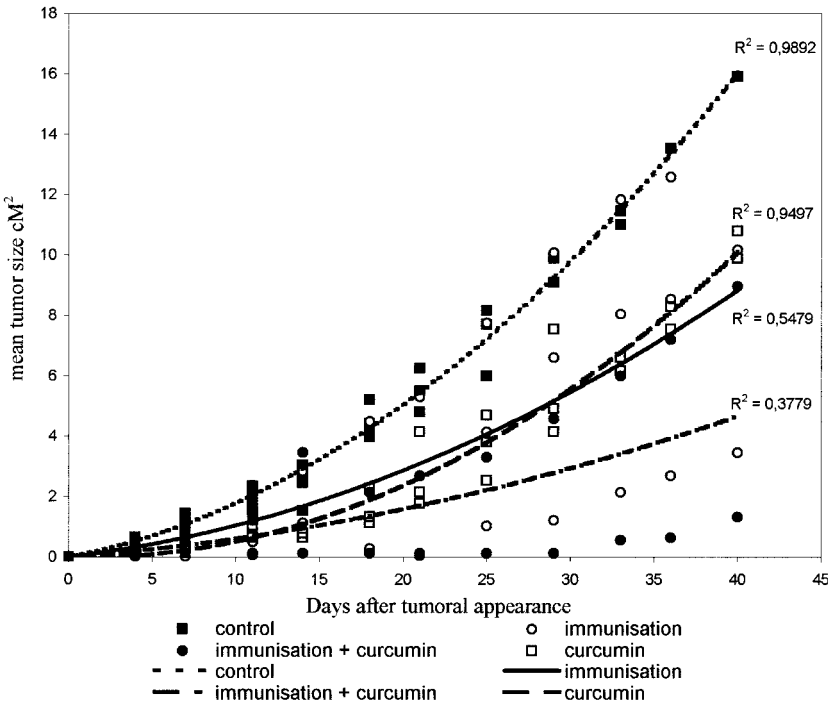

FIGURE 6 – Effect of curcumin on the growth of B16-R melanoma. B6D2F1 mice were injected s.c. with 1×10^6 viable B16-R cells. Control mice were untreated before and after tumor challenge (■). The immunized group received soluble B16-R proteins before viable B16-R injection (●). The curcumin group was treated each day with 25 mg/kg bw curcumin i.p. after tumoral appearance (□). The immunized + curcumin group received 4 injections of soluble proteins from B16-R cells before tumor challenge and were treated each day with 25 mg/kg bw curcumin i.p. when the melanoma was developed (■). The size of the tumors was assessed at least twice weekly and recorded as tumor area (in cM^2) by measuring the largest perpendicular diameters. Data are reported as tumor area for each mouse from each group (each dot represents a single mouse) and trend curves (polynomial regression calculated by the computer from the dots), show tumor growth of B16-R melanoma. The coefficients of determination (R^2) for each trend curve are shown on the right ($n = 6$ mice in all groups).

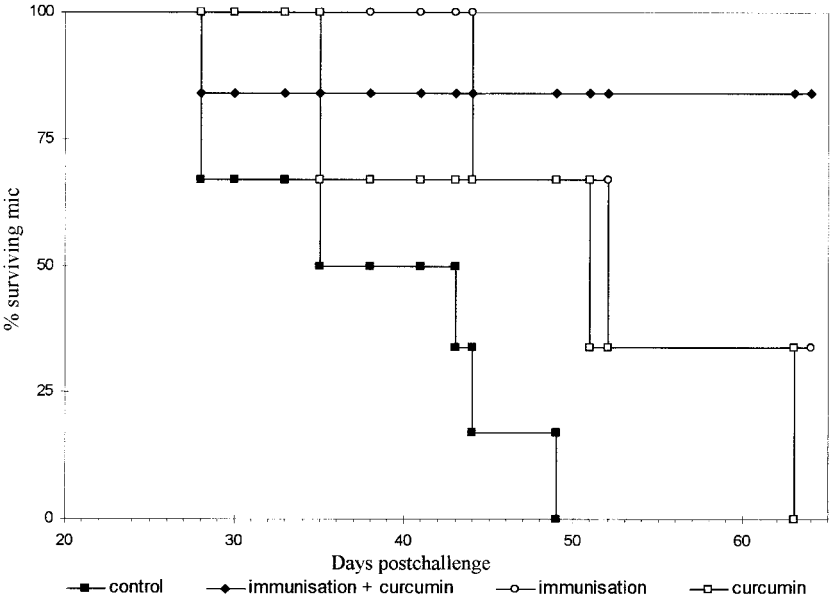

FIGURE 7 – Survival time of treated mice. B6D2F1 mice were injected s.c. with 1×10^6 viable B16-R melanoma cells. Mice bearing B16-R melanoma show the best enhanced survival when treated with immunization + curcumin. These mice were injected with soluble proteins collected from supernatant of B16-R cells before tumor challenge. They were then treated each day with 25 mg/kg bw curcumin after tumoral appearance (●). In the curcumin group, mice were treated each day with 25 mg/kg bw after tumoral appearance (□). In the immunized group, mice were only injected with B16-R soluble proteins before tumor challenge (○). In the control group, mice received no treatment before and after tumor challenge (■) ($n = 6$ mice in all groups).

zation with soluble B16-R proteins alone. Mice (5/6) receiving the combination treatment were still alive by Day 64 (Fig. 7). The median survival time of tumor-bearing animals was increased significantly in the treated group compared to untreated group. In the immunized + curcumin group, an increase in median survival time of more than 82.8% was observed. At a lower level, an increase in median survival time was also observed in curcumin alone-treated mice and immunization alone-treated mice (45.7% and 48.6%, respectively). No obvious side effects of curcumin were observed during the course of our investigations.

DISCUSSION

Curcumin has been studied for its wide-ranging effects on tumorigenesis,[25,26] angiogenesis[22] and signal transduction pathways.[23] We show *in vitro* that curcumin is cytotoxic for B16-R melanoma cells, cultivated as monolayers or 3-D cultures that simulate micrometastases.[34] Inhibition of Ehrlich ascites tumor (EAT) cell growth *in vivo* with corresponding reduction in cell number[29] validates the earlier findings that curcumin is an antineoplastic agent. It seems that curcumin has a cell specificity of action because it did not reduce mouse fibroblast (NIH3T3) cell number but decreased the number of EAT cells and human umbilical vein endothelial cells.[22] These effects are attributed to induction of apoptosis by curcumin *in vivo*[29] and *in vitro*.[22] Several authors report on an antiproliferative effect on colon cancer,[35] breast cancer cells[36] and in human leukemia cells.[37] In contrast, curcumin can inhibit apoptosis (such as in T lymphocytes) in other systems.[38] Somasundaram *et al.*[39] considered the possibility that

curcumin might decrease the effectiveness of anti-tumor drugs. They used breast cancer (both in tissue culture and *in vivo* models) as an example of the possible systemic effects of this molecule and reported that curcumin inhibited the pro-apoptotic activity of several chemotherapeutic agents, ROS generation, JNK activation and release of mitochondrial cytochrome *c*. Because of obvious discrepancies in results concerning the pro-apoptotic activity of curcumin[37,38,40] and in some cases conflicting results as observed in HT-29 human colon cancer,[32,35] we have studied whether the lethal effect of curcumin on B16-R was due to induction of programmed cell death. We have demonstrated that curcumin is capable of inducing apoptosis on B16-R cells (time- and dose-dependent increase in internucleosomal fragmentation of DNA). Our results agree with Bush *et al.*[41] showing that curcumin induces apoptosis in different melanoma cell lines independently of p53 and Bcl-2 family expressions but by activation of the death receptor Fas-initiated FADD/caspase-8-dependent apoptosis pathway. Pal *et al.*[29] demonstrate on Ehrlich's ascites carcinoma cells that curcumin induced tumor cell death by upregulation of the proto-oncoprotein Bax, releasing of cytochrome *c* from mitochondria and activation of caspase 3. These authors also observed that the tumor development caused suppression of host immune system in untreated tumor-bearing mice (depletion in splenic mononuclear cell) in comparison to normal mice. Interestingly, when tumor-bearing mice were treated with curcumin, their splenic cell number was reverted back to normal level showing a differential action of this molecule on tumor and normal cells of the host (curcumin activated tumor cell killing and restored tumor induced immuno depletion of the host). Such results have implications for the clinical use of curcumin in cancer immunotherapy. It has been shown by Gururaj *et al.*[22] that this active principle is an angio inhibitory compound. Because of these pro-apoptotic and anti-angiogenic properties, we have investigated the capacity of curcumin to elicit *in vivo* anti-tumor activity against the poorly immunogenic and highly metastatic chemoresistant B16-R cells.

Previous works have demonstrated that *in vivo* administration of curcumin (40–80 mg/kg bw) can inhibit tumor growth, tumor metastasis and significantly increase the life span on EAT cells[29] on skin carcinogenesis[42] and B16F-10 melanoma cells.[43,44] In contrast, Caltagirone *et al.*.[45] studied several *in vivo* polyphenolic compounds on the growth, invasive and metastatic potential of B16-BL6 melanoma cells and observed that daily curcumin treatment was ineffective on intramuscular melanoma growth at doses between 12.5–50 mg/kg bw. Surprisingly, we have observed *in vivo* a slight inhibition on B16-R melanoma growth when curcumin was used alone although we used 25 mg/kg bw. In this case, when no treatment was carried out, no humoral immune response could be detected against B16-R proteins. Unfortunately, all mice from the curcumin-treated group died within 2 months after tumor challenge. The median survival time was 51 days, however, which shows an increase in median survival time of 45.7% compared to untreated group. We assume that the slight inhibition effects observed in our study could be due to the anti-angiogenic properties of curcumin. This hypothesis is in agreement with the results of Gururaj *et al.*[22] who demonstrated *in vivo* an antiangiogenic effect with approximately the same dose (20 mg/kg bw) of curcumin. This action is attributed to downregulation of the expression of proangiogenic genes such as vascular endothelial cell growth factor (VEGF), angiopoietin 1 and 2 and kinase-insert domain receptor (KDR).[22] Moreover, random postmortem examination on mice treated daily with curcumin showed less lung metastases induced by B16-R cells than in untreated mice. This observation is in agreement with the anti-metastatic activity of curcumin demonstrated by Menon *et al.*[44] Because a slight inhibitory effect on tumor growth is observed in our study, we believe that higher doses (>20–25 mg/kg bw/day) could induce a total inhibition of growth or regression of melanoma and could prevent metastases *in vivo*. Based on the discrepancy of results on the *in vivo* anti-melanoma effects of curcumin (*i.e.*, Caltagirone *et al.*[45] *vs.* Menon *et al.*[44] and our work), we believe that the inhibitory property of this compound on tumor growth is dose- and cell type-dependent. Thus, the toxicology, pharmacokinetics and biologically effective doses of curcumin have to be studied. Somasundaram *et al.*,[39] using an *in vivo* cancer model have shown that 3.2 g/kg bw of curcumin significantly inhibited cyclophosphamide-induced tumor regression. From their findings, curcumin inhibits chemotherapy-induced apoptosis and additional studies are needed to determine whether cancer patients undergoing chemotherapy should avoid curcumin treatment and possibly, limit their exposure to curcumin-containing food. Curcumin has been found to be non-toxic in animals at concentrations of 0.5–2 g/kg[46] and up to 8 g/day in a Phase I chemoprevention clinical trial in humans.[47]

Pal *et al.*[29] have demonstrated *in vivo* that curcumin activated tumor cells killing and could ameliorate immunotoxicity due to tumor. As it is known that apoptotic tumor cells induce immune response,[48] we have combined a prophylactic treatment using simple soluble B16-R cell proteins with curcumin in B16-R tumor-bearing mice to test the anti-tumor activity improvement of our immunization. The results of our study seem to demonstrate that the combination therapy is more effective than individual therapy. Moreover, analysis of the sera from animals receiving the combination treatment showed a better humoral immune response than found in sera from mice in the immunized group. From this result, it can be assumed that curcumin could also activate anti-B16-R immunocompetent cells stimulated previously by *in vivo* immunization with soluble B16-R proteins. Consequent to the delay of tumor growth and the enhancement of the immune response in mice receiving the combination therapy, the life span of animals with tumor increased significantly (increase in median survival time is >82.8% *vs*. 48.6% and 45.7%, respectively for immunized group and curcumin-treated group).

This type of differential action of curcumin on tumor and normal cells of the host have been reported by Pal *et al.*[29] and seems to have much significance in the area of cancer therapy. Further investigations on toxicity, biological activities and molecular mechanisms of anti-tumor effect of curcumin should provide effective strategies against melanoma and a wide range of cancers.

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