MANGIFERA INDICA L. EXTRACT MODULATES NF-*KAPPA*B SIGNALING PATHWAY IN THE COURSE OF ACTIVATION-INDUCED T CELL DEATH

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Summary

Activation-induced cell death (AICD) plays an important role in maintenance of peripheral lymphocyte homeostasis. CD95 ligand (CD95L) expression is crucial in the induction of AICD. TCR engagement results in the activation of several transcription factors that cooperatively act on the CD95L promoter to induce de novo transcription and led to apoptotic process. It is well established that NF-κB is activated by oxidative signals produced during TCR signaling. *Mangifera indica* L extract has raised considerable interest because of its antioxidant properties. This extract has shown to protect T cells from *in vitro* AICD. In the present study, we investigated the contribution of NF-κB in the protective effect demonstrated by *M. indica* extract in T cells. Our results show, *M. indica* extract treatment did not prevent TCR-induced IκB degradation but significantly decreased the protein expression of NF-κB (p65). Our findings suggest that the T cell survival effect of *M. indica* extract on T cells is associated with its capacity to modulate NF-κB signalling.

Keywords: Mangifera; AICD; NF-kappaB

Abbreviations: AICD, activation-induced cell death; CD95L, CD95-ligand; DMSO, dimethylsulfoxide; M. indica, Mangifera indica L.; ROS, reactive oxygen species; TCR, T-cell receptor.

Introduction

For peripheral T cells, a particular form of apoptosis induced by repeated T cell receptor (TCR) stimulation, known as activation-induced cell death (AICD) contributes to maintain the immune system homeostasis (1). The imbalance in this apoptotic process leads to severe diseases (2). CD95 and its ligand (CD95L) play a crucial role in this type of cell death (3). TCR engagement triggers a complex intracellular signaling cascade that involved the generation of reactive oxygen species (ROS) and the increase in cytosolic Ca^{2+} influx (4). These signals result in the activation of several transcription factors that cooperatively act on the CD95L promoter to induce its *de novo* transcription and led to apoptotic process (5).

NF-κB transcription factors are key regulators of immune and inflammatory responses and they are also implicated in the control of apoptosis. They exist primarily as a p50/p65 heterodimer. Normally, NF-κB remains in the cytosol sequestered in an inactive state by the inhibitory proteins IκB. Many stimuli induce NF-κB activity, including tumor necrosis factor (TNF), activators of PKC, lipopolysaccharide (LPS), ionizing radiation and oxidants. Activation of T cells via TCR also leads NF-κB activation (6). These stimuli induce phosphorylation, ubiquitination and the subsequent degradation of IκB proteins, leading to the release of NF-κB which translocates to the nucleus to bind specific DNA sequences. Several NF-κB binding sites are localized at the human CD95L promoter (7).

The stem bark extract from *Mangifera indica* L. (Vimang), rich in polyphenolic compounds, has probed antioxidant activities (8). We have previously established *M. indica* extract protects T cell from *in vitro* AICD by a mechanism that attenuated TCR-induced early events, including ROS generation and intracellular free Ca^{2+}acumulation (9). In the present study, we investigated the role of NF-κB signaling in the protective effect demonstrated by *M. indica* extract in T cells.

Methods

Chemicals: Phytohemagglutinin (PHA) was obtained from Sigma (St. Louis, Missouri, USA). The monoclonal anti-CD3 Ab (OKT3) was provided by Dr. Karsten Gulow (German Cancer Research Center, Heidelberg, Germany). All cell culture supplies were obtained from GIBCO BRL (Invitrogen Life Technologies, Karlsruhe, Germany). Antibodies against β-actin were purchased from Abcam (Cambridge, United Kingdom).

Antibodies against IκB and phospho-IκB (p-IκB) were obtained from Cell Signaling Technologies (Beverly, MA, USA). Antibodies against NF-κB (p65) were obtained from. Santa Cruz Biotechnology (Santa Cruz, CA, USA).

The aqueous extract from *M. indica* was supplied by the Center of Pharmaceutical Chemistry (Cuba). The chemical composition of this extract has been characterized by chromatographic methods, mass spectrometry and UV–vis spectrophotometry (10). *M. indica* extract solutions were prepared by dissolving the powder in DMSO, at 100 mg/mL. Further dilutions were made in complete culture medium. The final concentration of DMSO in culture did not exceed 0.2% to prevent solvent influence on the results.

Cell culture and activation: Freshly isolated T cells from peripheral blood of healthy individuals were obtained from buffy coats and prepared by Ficoll-Paque (Pharmacia Diagnostic, Freiburg, Germany) density centrifugation as previously described (11). Human peripheral blood T lymphocytes were cultured in RPMI supplemented with 10% heat inactivated FCS in a humidified atmosphere (5% CO2 plus 95% air). For activation, freshly isolated T cells were cultured with mitogen (PHA, 1 μg/ml) for 16 h. Thereafter, T cells were washed and cultured in the presence of IL-2 (25 U/ml) for an additional six days as described previously (11) (then referred to as day 6 T cells).

Western blot analysis: Western blot analyses were carried out as described elsewhere. Day 6 T cells were treated for 4 hours without or with *M. indica* extract (200 μg/ml), and then stimulated for indicated times with PMA/ ionomycin (10 ng/ml/0.5 μM). The concentration of *M. indica* extract used in the experiments was according with the one reported protective in assays of AICD (9). Total soluble protein lysates were resolved in 8-12 % SDS-PAGE and were subjected to immunodetection with antibodies to IκB, p-IκB, NF-κB (p65) and finally β-actin for a loading control. Signal detection was carried out using the enhanced chemiluminescence system (Amersham). Densitometric analysis was performed using a laser scanner and bands were quantified in TIF images using densitometry analysis software (TotalLab 1D Gel Analysis v2003.02, Amersham Biosciences).

Results

Effect of *M. indica* extract on NF-κB protein level upon PMA/ionomycin-stimulation

To study the effect of *M. indica* extract on NF-κB protein level we assessed a semiquantitative analysis of kinetic western blots. Day 6 T cells were pretreated

without or with *M. indica* extract and then stimulated with PMA/ionomycin for indicated time. We observed at all time point tested, PMA/ionomycin induced any significant change on total NF-κB (p65) protein level, neither in -untreated nor *M. indica*-treated cells. A significant decrease in the levels of NF-κB (p65) protein expression was observed in *M. indica*-treated comparing with -untreated cells (Fig. 1A, B).

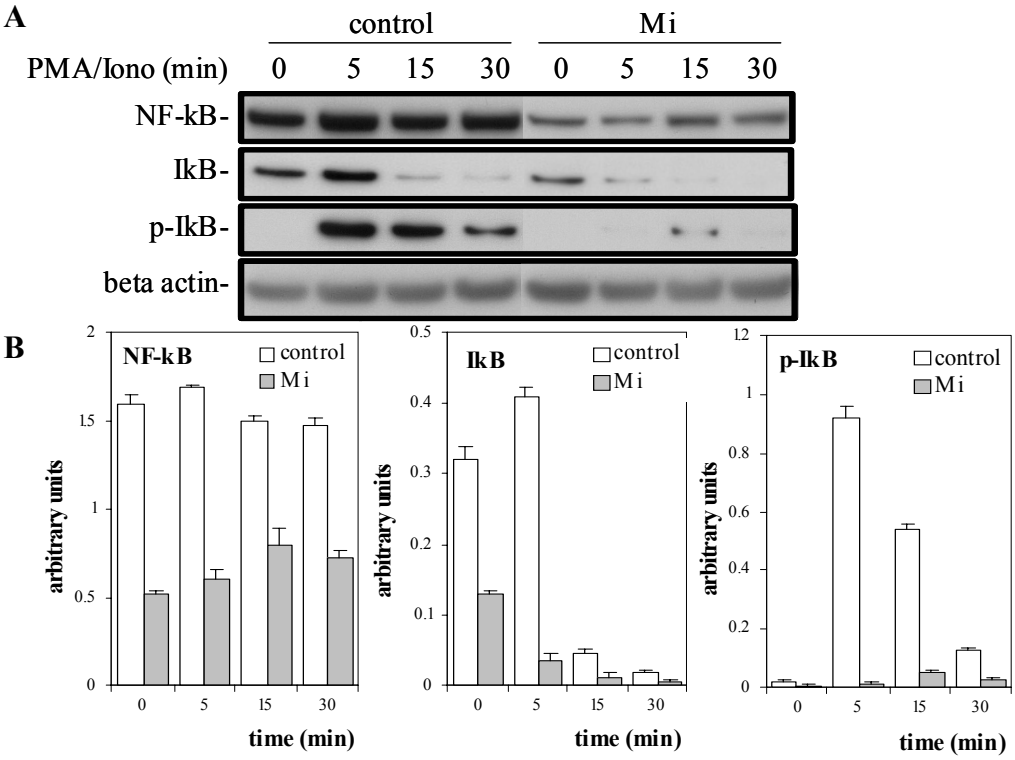

Figure 1. Effect of *M. indica* extract on NF-κB signaling pathway in primary human T cells. (A) Day 6 T cells, either untreated (left panel) or pretreated (right panel) with 200μg/mL *M. indica* extract (Mi) were stimulated with PMA/ionomycin (10 ng/ml/1 μM) for different times. Whole cell extracts were prepared and immunoblotted with Abs against NF-κB, IκB, phospho-IκB and β-actin. (B) Protein levels were quantified by densitometric analysis and expressed with respect to the value for β-actin as the standard protein. Bars show means (n=3) + SE of (Band volume of NF-κB, IκB or p-IκB)/(Band volume of corresponding of β-actin).

Effect of *M. indica* extract on IκB phosphorylation and protein degradation upon PMA/ionomycin-stimulation

To evaluate the effect of *M. indica* extract on IκB degradation and phosphorylation after PMA/ionomycin-stimulation we used the same conditions of above described experiment. *M. indica*-untreated cells showed the phosphorylation and degradation of IκB upon PMA/ionomycin-stimulation. *M. indica*-treated cell showed a similar kinetic of both events although the phosphorylation was nearly undetectable. A significant decrease in the levels of IκB protein expression was observed in *M. indica*-treated comparing with -untreated cells. (Fig. 1A, B).

Discussion

Our previous results have established that *M. indica* extract attenuates TCR-induced increased of ROS and intracellular free Ca^{2+} and consequently, downregulates CD95L mRNA expression and AICD. The mechanism implicated should interfere with different transcriptional factors (NF-κB, AP-1, NF-AT), contributing to inhibit apoptotic process. In the present study, we have demonstrated *M. indica* extract modulates NF-κB signaling in the course of AICD.

It has been reported *M. indica* extract prevented TNF-induced IκB degradation and the binding of NF-κB to the DNA in T cells (12). A different study shows *M. indica* extract decreased mRNA levels of NF-κB but did not affect expression of IκB in mouse macrophages stimulated *in vitro* with LPS and interferon-γ (13). On this regards, it was found mangiferin, the main component of *M. indica* extract, inhibit the mRNA expression of p65 (14). It indicates *M. indica* extract modulates transcriptional events on NF-κB pathway.

Since we measured total protein levels the effect on protein expression could be assessed. In line with previously published results, we found *M. indica* extract decreased constitutive protein expression of NF-κB (p65) and consequently reduced its expression upon TCR-activation induced signals in T cells. On the other hands, differing with the literature, we found in our cellular system *M. indica* does not interfere with IκB degradation induced upon T-cell activation signals. However, low level of IκB was detected even before the stimulus and consequently phosphorylation was nearly undetectable. It has been demonstrated p65 and IκB are linked in an autoregulatory loop, ensuring that NF-κB is held in the cytoplasm until cells are specifically induced to translocate it to the nucleus (15).

We assume the inhibitory effect of *M. indica* extract on NF-κB protein levels induce a concomitant inhibition on IκB protein expression.
Taking into account that NF-κB is activated by oxidative signals produced after TCR triggering; the reduction of TCR-induced ROS generation previously shown by *M. indica* extract could interfere with pathways that involve NF-κB-mediated signal transduction. In consequence, this effect could contribute to suppress CD95L expression and attenuates AICD. In summary, our findings suggest the T cell survival effect of *M. indica* extract on T cells is associated with its capacity to modulate NF-κB signaling.

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