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Hepatocarcinogenic potential of the glucocorticoid antagonist RU486 in B6C3F1 mice: effect on apoptosis, expression of oncogenes and the tumor suppressor gene *p*53

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Abstract

Background: Glucocorticoids inhibit hepatocellular proliferation and modulate the expression of oncogenes and tumor suppressor genes *via* mechanisms involving the glucocorticoid receptor. Glucocorticoids also produce a receptor-mediated inhibitory effect on both basal and hormone-stimulated expression of a newly discovered family of molecules important for shutting off cytokine action. We therefore hypothesized that inhibiting glucocorticoid receptors may disturb hepatocellular growth and apoptosis. Consequently, we investigated the effect of RU486, a potent antagonist of the glucocorticoid receptor, on basal levels of hepatocellular proliferation and apoptosis in male B6C3F1 mice. Furthermore, we evaluated the effect of this compound on cellular genes involved in the regulation of these important processes.

Results: Data show that treatment of male B6F3C1 mice with RU486 (2 mg/kg/d, ip) for 7 days dramatically inhibited liver cell proliferation by about 45% and programmed hepatocellular death by approximately 66%. RU 486 also significantly increased hepatic expression of the oncogenes mdm2 and JunB, while reducing that of the tumor suppressor gene p53.

Conclusion: Exposure to RU486 may ultimately enhance the susceptibility of the liver to cancer risk by diminishing its ability to purge itself of pre-cancerous cells *via* apoptosis. This effect may be mediated through increases in the hepatic expression of the oncogene mdm2, coupled with decreases in that of the tumor suppressor gene p53. The decrease in hepatocellular proliferation caused by RU 486 may be related to effects other than its anti-glucocorticoid activity.

Background

RU486 (Mifepristone) has shown remarkable antiglucocorticoid and antiprogestrone activities both *in vitro* and *in vivo* [1,2]. This compound has a high affinity for ligand binding sites of both receptors [1,2]. Current clinical uses of RU486 are limited to its function as a progestrone antagonist [1,2], where it was approved in France in 1988, and recently in the USA as a means to induce early termination of pregnancy [1–3].

Based on its antiprogesterone activity, RU486 has been evaluated as a useful anticancer agent in organs sensitive to this hormone [4]. Although evidence was not conclusive in these studies, RU486 demonstrated a benefit to humans suffering from meningioma, breast cancer and

uterine leiomyomata [4]. Several *in vitro* studies also demonstrated that RU486 induced antiproliferative effects against murine mammary tumors [5] as well as cultured human endometrial stromal and epithelial cells [6,7].

Antagonizing the effects of glucocorticoids by RU486 may, however, result in deleterious effects. Glucocorticoids produce a receptor-mediated inhibitory effect on both basal and hormone-stimulated expression of a newly discovered family of molecules known as suppressors of cytokine signalling [8]. These molecules play an important role in shutting off cytokine action [8], and RU486 may therefore augment cytokine-mediated effects. Indeed, since tumor necrosis factor *alpha* (TNFα) is known to suppress hepatic apoptosis [9], a function which is considered a safeguard mechanism against neoplasia [10,11], the reported ability of RU486 to elevate hepatic TNFα production *in vivo* and to enhance cell sensitivity to its toxic effects is alarming [12]. Glucocorticoids also inhibit hepatocellular proliferation [13–15] and modulate the expression of oncogenes and tumor suppressor genes [11], *via* mechanisms involving the glucocorticoid receptor [16–18], further supporting the notion that inhibiting these receptors may result in harmful effects.

We tested the hypothesis that inhibition of the glucocorticoid receptor will trigger events that may ultimately lead to liver cancer. Consequently, we investigated the impact of RU486 on hepatocellular proliferation and programmed cell death in livers of male B6C3F1 mice, and quantified hepatic levels of genes involved in these events.

Results

Effect of RU 486 on hepatocellular proliferation and apoptosis

In control mice, 1.55 ± 0.12% of hepatocytes were labeled with BrDU (Fig. 1A). Treatment with RU 486 significantly diminished these labeling indices by approximately 45% to 0.86 ± 0.039% (Fig 1A). Similarly, RU 486 caused a remarkable decrease in programmed cell death in the liver. In control animals, 2.68 ± 0.7% of hepatocytes were undergoing apoptosis (Fig 1B). These levels were significantly reduced by 66% to 0.9 ± 0.16% in livers of mice pretreated with RU 486 (Fig 1B).

Increasing oncogene mRNA expression by RU 486

Pretreatment of mice with RU 486 caused significant increases in hepatic levels of JunB and mdm2 (Fig 2). A remarkable 2.5-fold increase in the mRNA of JunB was detected upon treatment with RU 486 (Fig 2A). RU 486 also increased the hepatic mRNA levels of the oncogene mdm2 by 21 ± 7% (Fig 2B).

Decreasing Liver mRNA Levels of c-myc and p53 by RU486

In contrast to its effect on JunB and mdm2 mRNA levels, RU 486 diminished the hepatic expression of c-myc and p53 mRNAs to comparable degrees (Fig 3). While mRNA of c-myc was significantly decreased by approximately 25 ± 3% (Fig 3A), that of p53 was also diminished significantly by about 21 ± 5% in the livers of animals treated with RU 486, compared to control mice (Fig 3B).

Discussion

Our results indicate that exposure to the anti-glucocorticoid antagonist RU 486 may enhance the potential for hepatocellular cancer. This conclusion is based on the observed inhibitory impact of RU 486 on programmed liver cell death.

Effect of RU 486 on liver cell proliferation

Glucocorticoids have been shown in various studies to inhibit DNA synthesis [13–15]. Therefore, we hypothesized that blocking the glucocorticoid receptor by the potent antagonist RU 486 would result in increased incorporation of BrDU into hepatocyte DNA, indicating an enhanced cellular proliferative capability. Indeed, RU486 caused a remarkable 2.5-fold increase in the expression of junB mRNA (Fig 2A). The protein product of this gene is a member of the activating protein-1 (AP-1) family [19], which upon binding to either c-fos or fos B protein form a complex that have high affinity to AP-1 binding concensus sequence [20]. A wide variety of chemicals, including growth factors, and tumor promotors increase the expression of this gene in various cell types [21]. Compared to untransfected keratinocytes, JunB-transfected cells grew more rapidly, and were highly invasive in an *in vitro* assay [22]. Therefore, the increased hepatic levels of junB gene, in mice treated with RU486, was expected to reflect an uncontrolled hepatocellular growth in these animals. Surprisingly, however, RU 486 diminished the capacity of liver cells to proliferate (Fig 1A). In order to explain this discrepancy, it is noteworthy to mention that unrelated to its anti-glucocorticoid activity, RU486 also has antioxidant properties that have been suggested to play a role in the inhibition of cell growth caused by this compound [7]. Such an effect and/or other anti-glucocorticoid-independent influences by RU 486 appears to prevail in relation to the overall impact of this compound on cell proliferation, and may indeed suggest, at a first glance, a protective effect against the risk of hepatocellular cancer.

Diminishing liver capacity to eliminate pre-cancerous hepatocytes

Despite the decrease in hepatocellular proliferation caused by RU 486, inhibition of hepatic apoptosis in animals treated with this compound (Fig 1B) represents a potentially deleterious effect. Apoptosis is considered a safeguard that enables an organ to purge itself of

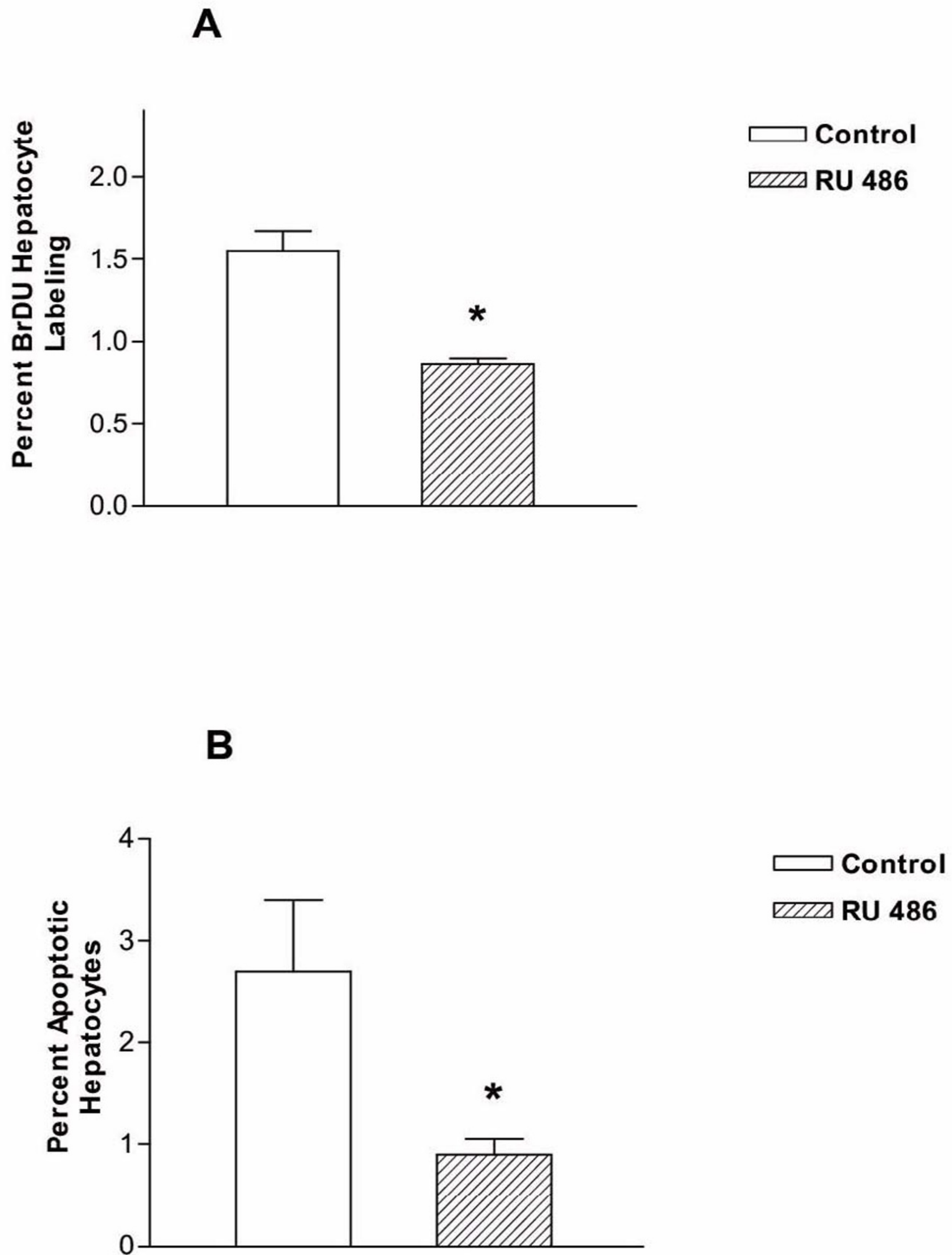

Figure 1
Hepatocellular proliferation (A) and apoptosis (B) in response to RU486. Mice were treated, and cell proliferation as well as apoptotic cells were quantified as described under Methods. Data are means ± SEM from 4–6 animals per group.

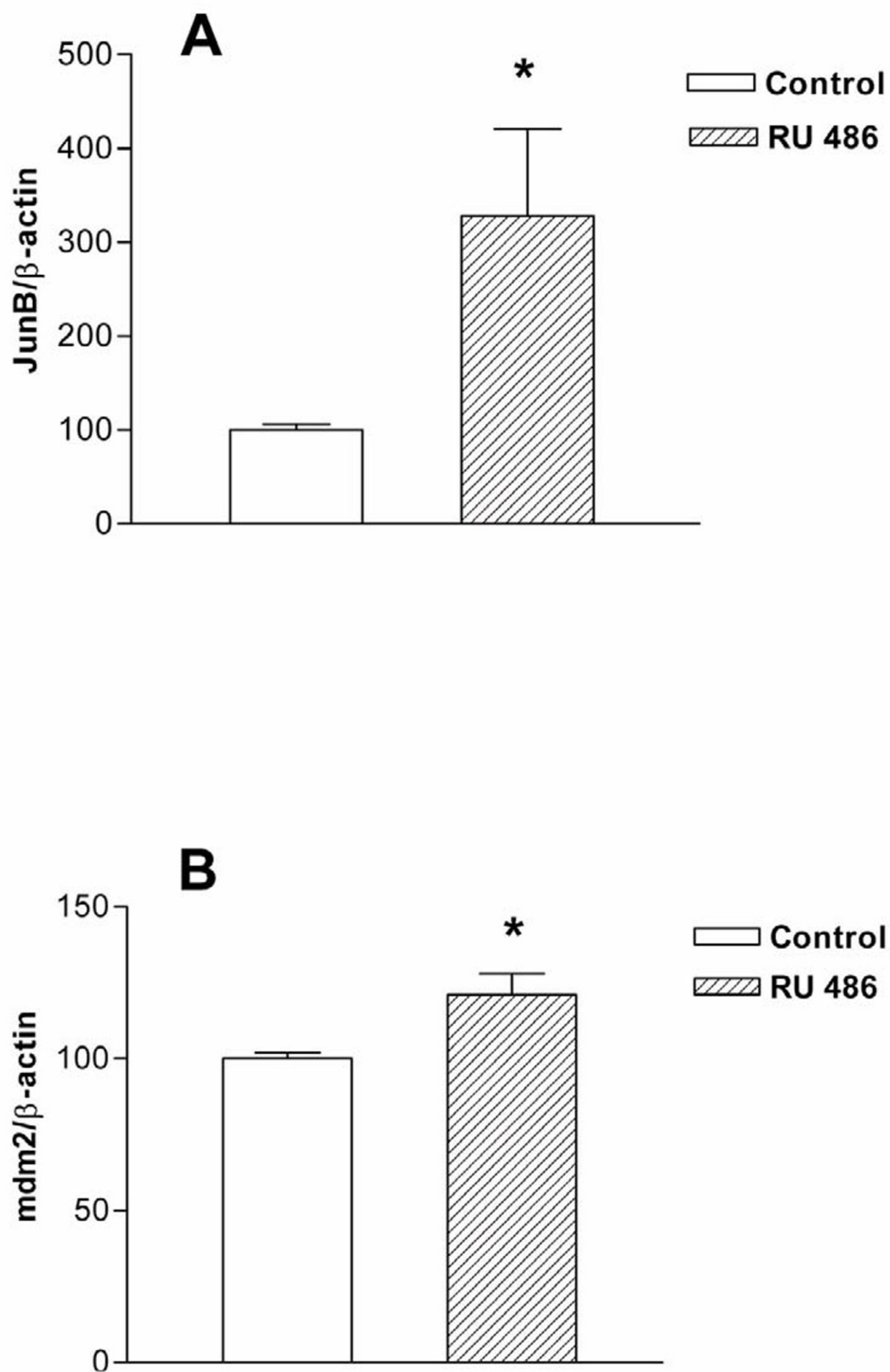

Figure 2
Elevated expression of JunB (A) and mdm2 (B) genes in livers of treated animals. Mice were treated and hepatic levels of JunB, mdm2 and β-actin were determined as described under Methods. Data are means ± SEM from 3–5 animals per group.

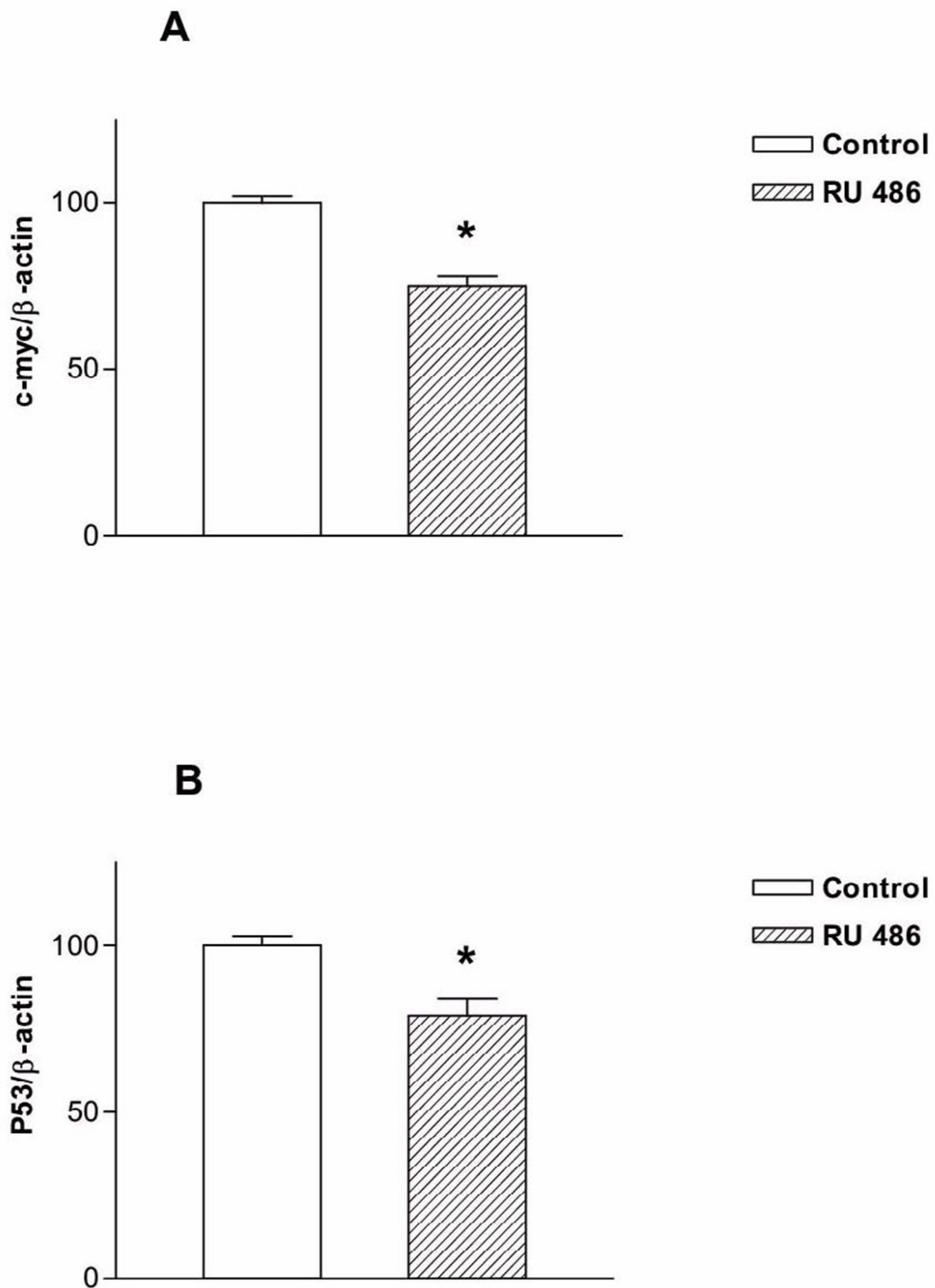

Figure 3
Diminished expression of c-myc (A) and p53 (B) genes in livers of treated animals. Mice were treated and hepatic levels of c-myc, p53 and β-actin were determined as described under Methods. Data are means ± SEM from 3–5 animals per group.

transformed cells [10,11], and its inhibition is proposed as a mechanism by which non-genotoxic hepatocarcinogens produce liver cancer [9,23]. We examined the effect of RU 486 on the hepatic expression of two genes involved in the regulation of this function [10,11], c-myc and p53, to evaluate how RU486 might inhibit apoptosis. This compound diminished the hepatic mRNA expression of both c-myc and p53 significantly (Fig 3). We previously reported that enhanced liver apoptosis in response to the antihepatocarcinogen rotenone was preceded and accompanied by a marked increase in the hepatic levels of c-myc mRNA [10], a proto-oncogene that plays a dual role in cell proliferation and apoptosis [24]. Since in our study [10] rotenone inhibited hepatocellular proliferation while enhancing apoptosis, we concluded that the increase in c-myc mRNA expression acted as a pro-apoptotic signal in the liver. Also, p53 modulates a battery of downstream genes involved in growth control and apoptosis [25,26]. Taken collectively, diminishing liver mRNA levels of both c-myc and p53 by RU 486 may help define the underlying reasons for the observed anti-apoptotic activity of this compound.

In addition to its effects on mRNA of c-myc and p53, RU486 significantly increased that of the oncogene mdm2 by approximately 20% (Fig 2B). The protein product of the mdm2 oncogene is over-expressed in a considerable number of tumors [27]. This protein is a pivotal negative regulator of p53 [27]; it interacts with the P53 protein masking its transcriptional domain and accelerating its proteasomal degradation [21,27]. The later process involves the deacetylation and ubiquitylation of the p53 protein by mdm2 [28]. Inactivation and/or degradation of p53 diminishes the cell ability to undergo apoptosis [21]. Conversely, p53 may influence mdm2. Over-expression of homeodomain-interacting protein kinase 2 (HIPK2), which increases p53 protein expression and/or stability, leads to a downregulation of mdm2 protein, but not mRNA [29]. Consequently, the RU486-induced increase in mdm2 mRNA expression, coupled with a decrease in that of P53, may also act in tandem to suppress the liver apoptotic capacity (Fig 1B).

NF-κB and the glucocorticoid receptor

The transcription factor NF-κB plays a critical role in controlling expression of downstream target genes in immune and inflammatory responses, development, and apoptosis [30]. Important advances have been made in understanding the signal transduction cascades that control NF-κB activation. Several lines of evidence indicate that NF-κB is constitutively activated in cancer cells, suggesting that it plays an important role in tumorigenesis [30]. Activated glucocorticoid receptors have been shown to repress NF-κB-mediated gene expression [31], and to cause a significant reduction in nuclear levels of p65 protein, a subunit of NF-κB [31]. A variety of mitogens, cytokines, and reactive oxygen intermediates have been shown to rapidly cause the translocation of NF-κB to the nucleus [30–32], and the ability of dexamethasone to interfere with this translocation is blocked by prior treatment with the glucocorticoid antagonist RU 486 [30]. Based on these findings, it is plausible to speculate that exposure to RU 486 may interfere with the function of endogenous glucocorticoids as regulators of cell cycle, allowing the initiation and progression of malignant transformation of liver cells.

Other mechanisms that may participate in the RU 486-induced inhibition of hepatic apoptosis include the fact that this compound blocks the inhibitory effect of glucocorticoids on suppressors of cytokine signalling in the liver [8]. This family of proteins is induced by growth hormones and cytokines, and is strongly repressed *in vivo* by glucocorticoids [8]. Up-regulation of these proteins by RU 486 [8], coupled with significant increase in hepatic TNFα level found in animals treated with RU 486 [12], may contribute to the inhibition of apoptosis observed in our study (Fig 1B). Increased liver production of TNFα has been promoted as a significant factor in the mechanism by which non-genotoxic hepatocarcinogens produce their effect [9].

Conclusion

RU 486 has recently become available in the USA for clinical use as an effective non-surgical means to induce abortion. The inhibitory effect of this compound on hepatocellular apoptosis, observed in this study, raises questions about the potentially deleterious impact of RU 486. A single dose of RU 486 such as that recommended for abortion, or possible long-term treatment with this drug in the future for other therapeutic purposes could potentially initiate events that could increase the risk of hepatic cancer. Such an effect would be of special concern for individuals concurrently exposed to other carcinogens.

Methods

Chemicals and supplies

TRI REAGENT was the product of Sigma Chemical Co, St Louis, MO, while MAXI script™ labeling kit and the Ribonuclease Protection Assay kit were purchased from Ambion, Inc, Austin, TX. ^{32}P UTP was obtained from NEN Life Science Products, Inc, Boston, MA. ApopTag™ kit was purchased from Intergen (Purchase, NY). Probes for mdm2, JunB, c-myc and β-actin genes were also obtained from Ambion, lnc. (Austin, TX).

Animal treatment

Male B6C3F1 mice (Charles River, Portage. Michigan) weighing 20–25 g were maintained on a daily cycle of alternating 12 hours periods of light and darkness. Mice

received the glucocorticoid antagonist RU486 (2 mg/k/day, ip), or a corn oil vehicle for 7 days. Care and handling of animals were in accordance with the guidelines of the USDA through the Animal Welfare Act Guide for the Care and Use of Laboratory Animals, Department of Health and Human Services Publication No 85–23.

Ribonuclease protection assay (RPA)

Hepatic RNA was extracted and quantified as we reported previously [10]. RNA was mixed with ^{32}P-labeled c-myc, mdm2, p53, JunB, and β-actin probes, then co-precipitated, purified, and electrophoresed on 5% polyacrylamide gel. Following electrophoresis, gels were exposed to X-ray film overnight. Band intensities were quantified using Molecular Analyst software and a GS-250 Molecular Imager (Bio-Rad Laboratories, Hercules CA).

Hepatocellular proliferation and apoptosis

Bromodeoxyuridine (BrDU) incorporation into DNA, a measure of cell proliferation, and enzymatic end labeling of 3'-hydroxy-DNA strand breaks, a measure of apoptosis, were quantified *in situ* as we have reported previously [10].

Statistical analysis

Means of the two experimental groups were compared using the Student t-test. Statistical significance was defined as $p < 0.05$. All data reported are means ± SEM of 3–6 mice per group.

Authors' contributions

JY performed the cell proliferation and apoptosis assays, and carried out related analyses. MB conceived, designed and coordinated the study. Both authors participated in writing the manuscript.

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