Inhibition of Hepatocarcinogenesis by ArtinM via Anti-proliferative and Pro-apoptotic Mechanisms

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Abstract. ArtinM is a d-mannose-binding lectin found in the seeds of Artocarpus heterophyllus (jackfruit) that interacts with N-glycans, that is associated with receptors on the surface of phagocytic cells and induces the production of inflammatory mediators. Some of them are especially important because they may be required for antitumor immune response. This study aimed to evaluate the effect of ArtinM on hepatocellular preneoplastic foci. Wistar rats received 50 mg/kg of diethyl-nitrosamine (DEN) intraperitoneal weekly for 12 weeks. From the 14th week, the treated animals received 50 μg/kg of ArtinM subcutaneous every 2 weeks until the 18th week, whereas control animals were injected with vehicle alone. Preneoplastic-related factors were estimated using histological, western blotting and RT-PCR analysis. In comparison to the groups exposed to DEN, the ArtinM-treated rats showed diminution of preneoplastic foci, decreased expression of proliferating cell nuclear antigen (PCNA), increased number of nuclear p21 and p27 stained cells, augmented number of apoptotic cells, increased expression of p53, p42/44 MAPK and p21 proteins, reduced cyclin D1 (CCND1) protein levels and increased expression of TNFα and IFNγ genes. No difference was observed in interleukin 12 (IL12) protein levels. These findings indicate that ArtinM may provide protection against hepatocarcinogenesis as a result of the induction of cell-cycle blockage and pro-apoptotic mechanisms.

Hepatocellular carcinoma (HCC) represents the vast majority of primary hepatic malignancies. HCC is also the fifth leading cause of cancer worldwide and the third most common cause of death among cancer patients (1). The molecular pathogenesis of HCC is complex and can arise from a normal liver, an altered non-cirrhotic liver or from a cirrhotic liver, the latter of which corresponds to almost all HCC cases (2). A variety of risk factors have been associated with HCC, such as hepatitis B and C, vinyl chloride, tobacco, aflatoxin B1, chronic alcohol consumption, non-alcoholic fatty liver disease, hemochromatosis (3) and exposure to chemical carcinogens (4).

Hepatocarcinogenesis is a multifactorial process during which extrinsic stimuli induce genetic alterations in mature hepatocytes, recruiting them to a successive proliferative cycle and death until the establishment of a population of monoclonal cells (5). HCC may arise from preneoplastic lesions where accumulation of genetic alterations secondary to cellular stresses imposed by carcinogens or other factors together with an inherent high proliferative capacity and survival of hepatocytes, promote malignant transformation and carcinogenesis (6). However, the development of preneoplastic lesions in clinically detectable HCC can take a long time (7). This exceptional characteristic of HCC has motivated studies on the chemoprevention of hepatocarcinogenesis rather than on curative strategies. Therefore, the focus on the development of substances capable to prevent preneoplastic foci progression is relevant. Lectins are carbohydrate-binding proteins or glycoproteins of non-immune origin that recognize and reversibly bind to glycans without altering their covalent structure. Plant lectins are important tools in cell biology and immunology because they show potential for clinical application (8, 9). Lectins can identify glycan determinants that are markers of clinical interest and possess antitumor and anti-carcinogenic properties that could be useful in the development of cancer.
In accordance with a previous protocol of hepatocarcinogenesis induction, efforts were made to minimize animal suffering and to reduce the number of temperature and in 12:12 h light/dark cycles, with food provided. 250-300 g were housed in individual cages under standard conditions under sterile condition to a final concentration of 50 μg/ml in an isotonic and buffered (pH 7.4) solution. The vehicle alone was used as a control preparation.

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Recently, extracts isolated from Artocarpus heterophyllus (jackfruit) reported to show anti-proliferative properties on human cancer cell lines and murine B-cell lymphoma (11, 12). ArtinM is a d-mannose-binding lectin found in the seeds of Artocarpus heterophyllus that interacts with N-glycans and is associated with receptors on the surface of phagocytic cells to induce production of inflammatory mediators, such as tumor necrosis factor alpha (TNFα) (13), interleukin 12 (IL12) and interferon gamma (IFNγ) (14). Moreover, the protective effect of ArtinM against intracellular pathogens (15, 16, 17) and against the development of hematopoietic cancer (18) is well-known. However, it is necessary to investigate the effects of ArtinM against neoplastic progression in vivo. Therefore, we evaluated the effect of ArtinM in hepatocarcinogenesis on rats.

Materials and Methods

Extraction and purification of ArtinM. The lectin ArtinM was extracted from jackfruit (Artocarpus heterophyllus) seeds and purified by affinity chromatography as previously described (19). In brief, dried seeds from Artocarpus heterophyllus were ground and suspended 10% (w/v), in 10 mM PBS, pH 7.2, for 24 h at 37°C. After centrifugation, supernatant fraction (crude extract) was dialyzed and depleted of others glycoproteins by at least three successive adsorption procedures on a 5-ml settled bed of D-galactose-agarose. The lectin homogeneity was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis (SDS-PAGE) and protein concentration was measured using the BCA kit (Sigma-Aldrich, MO, USA). Purified lectin was diluted under sterile condition to a final concentration of 50 μg/ml in an isotonic and buffered (pH 7.4) solution. The vehicle alone was used as a control preparation.

Animals and experimental protocol. Male Wistar rats (n=30), weighing 250-300 g were housed in individual cages under standard conditions of temperature and in 12:12 h light/dark cycles, with food provided ad libitum. Animal management procedures conformed to the International Guiding Principles for Biomedical Research Involving Animals. The rationale, design and methods of this study were approved by our institutional animal ethics committee (n°90/2009). Efforts were made to minimize animal suffering and to reduce the number of animals used. The experimental design was performed in accordance with a previous protocol of hepatocarcinogenesis induction in rats (20). The animals were randomly allocated into three groups. The control group (Ct) (n=6) received the vehicle (distillated H2O) for diethyl-nitrosamine (DEN) weekly for 12 weeks and from the 14th week received the vehicle (distillated H2O) for ArtinM for 6 weeks. The DEN group (D) (n=12) was administered with DEN (Sigma-Aldrich, 50 mg/kg) intraperitoneally, weekly, for 12 weeks. Beginning at the 14th week, they received the ArtinM vehicle subcutaneously, once every 2 weeks for a total of 3 doses, until the 18th week. The DEN+ArtinM (D+AM) (n=12) group followed the same pattern as the DEN group, but received ArtinM (50 μg/kg) added to the vehicle. At the 20th week, the animals were sacrificed under anesthesia and liver samples were collected. The hepatic fragments were frozen in liquid nitrogen and stored at −70°C or fixed in 10% phosphate-buffered formalin for 24 h at room temperature.

Histological analysis. Paraffin-embedded liver sections were stained with hematoxylin and eosin (H&E) for the evaluation of the preneoplastic lesions. The number of preneoplastic foci was counted randomly in at least 30 representative high-power fields (40×) by two of the authors (MMB and LZR). All discordant results were resolved using a multi-head microscopic analysis system. Diagnosis of preneoplastic foci was considered when obvious cellular alterations were observed in a well-defined group of hepatocytes, such as large cells with abundant cytoplasm, increased nuclear/cytoplasmic ratio, enlarged pleomorphic nuclei, clumped chromatin, thick nuclear membranes and prominent nucleoli (21). For immunohistochemical analysis, 4-μm-thick sections mounted on poly-L-lysine coated slides were deparaffinized, rehydrated, immersed in 10 mmol/L citrate buffer, pH 6.0 and submitted to heat-induced epitope retrieval using a vapor lock for 45 min. The slides were briefly rinsed with phosphate-buffered saline (PBS) and immersed in 3% hydrogen peroxide for 20 min to block endogenous peroxidase. Non-specific protein binding was blocked with normal serum (Vectastain Elite ABC Kit, Universal, Vector Laboratories Inc., Burlingame, CA, USA) for 30 min. The sections were then incubated with monoclonal primary antibodies anti-proliferating cell nuclear antigen (PCNA) (PC10, dilution 1:500, DAKO AS, Glostrup, Denmark), anti-p21 (SX118, dilution 1:100, DAKO) and anti-p27 (SX53G8, dilution 1:100, DAKO) for 2h at room temperature in a humid chamber. Following washes in PBS, biotinylated pan-specific universal secondary antibody (Vectastain Elite ABC Kit) was applied for 30 min. Next, the slides were incubated with the avidin–biotin–peroxidase complex (Vectastain Elite ABC Kit) for 30 min and developed with 3,3-diarnimo-benzidinetetrahydrochloride (DAB) (Vector Laboratories Inc.) in PBS for 5 min. The slides were counterstained with Harris’s haematoxylin, dehydrated and mounted with Permount (Biomeda, Foster City, CA, USA). As negative controls, all specimens were incubated with an isotope-matched control antibody under identical conditions. The immunolabelling percentage was evaluated by a ratio of unequivocal labeling at nucleus (PCNA) or cytosol (p21 and p27) at each 100 counted preneoplastic foci. Additionally, paraffin-embedded liver tissue sections, deparaffinized and incubated with 20 g/ml proteinase K (Promega Corp., Madison, WI, USA) were analysed for transferase-mediated dUTP-biotin nick-end labeling (TUNEL) using a DeadEnd peroxidase in situ apoptosis detection kit (DeadEnd TUNEL; Promega). In brief, samples were treated with 3% hydrogen peroxide to quench endogenous peroxidase activity. After adding equilibration buffer, sections were treated with terminal deoxynucleotidyl transferase and digoxigenin-dNTPs for 60 min at 37°C. Specimens...
were then treated with anti-digoxigenin-peroxidase for 30 min at 37°C, colorized with DAB, and counterstained with Harris’s haematoxylin. Finally, slides were rinsed, dehydrated, and mounted. A negative control for non-specific incorporation of nucleotides or binding of enzyme-conjugate was prepared by omitting the terminal deoxynucleotidyl-transferase enzyme. The number of TUNEL-positive preneoplastic foci cells was evaluated randomly in at least 30 representative high-power fields (×40) per sample and mean values were obtained. The cells were considered positive when obvious brown nuclear staining was detected.

Protein quantification by Western blotting. The protein levels of p53, p21, cyclin D1, and p42/44 MAPK (mitogen activated protein kinase) were quantified by Western blotting. Cell extracts were obtained from frozen liver samples using a Triton-based lysis buffer containing protease and phosphatase inhibitors. The protein samples (25 mg) were resolved on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies against p53 (Ab-1, dilution 1:50, Calbiochem, Cambridge, MA, USA), p21 (SX118, dilution 1:100, DAKO), cyclin D1 (P2D1F11, dilution 1:200, Novocastra, Newcastle, UK), p42/44 MAPK (20G11, dilution 1:1000, Cell Signaling Technology, Danvers, MA, USA) and β-actin (sc-81178, dilution 1:400, Santa Cruz Biotechnology Inc., Heidelberg, Germany) as a loading control. Next, the membranes were washed and incubated with a peroxidase-conjugated secondary antibody. The proteins were detected by electrochemiluminescence (ECL, Amersham Biosciences Corp. Piscataway, NJ, USA). The resulting blots were scanned using an ImageReader LAS-3000 imaging densitometer (Fujifilm, Tokyo, Japan), and the optical densities of the specific protein bands were quantified using the ImageGauge software (Fujifilm). Ponceau Red staining of the crude homogenates on the membranes was used to determine equal loading/transfer across the lanes.

Gene expression analysis by real time PCR (RT-PCR). Frozen hepatic sample were used to quantify the genic expression of TNFα, IL12 and IFNγ. The tissue fragments were homogenized and submitted for analysis of the expression of total RNA using a retro-transcription kit (Ready-To-Go You-Prime First-Strand Beads and pd(N)6 Random Hexamer as primers (Amersham Biosciences). mRNA amplification, with concurrent quantification, was conducted by RT-PCR StepOnePlusTM (Applied Biosystems Inc., Foster City, CA, USA) using specific primers to TNFα, IL12, IFNγ and 18S RNA transcripts (Assays-on-Demand Gene Expression Products, Applied Biosystems) and a specific Taq polymerase enzyme (TaqMan Universal PCR Master Mix, No AmpErase UNG - 2X, Applied Biosystems). All experiments were performed in duplicate and the results were normalized to 18S rRNA expression.

Statistical analysis. Data were analyzed using the GraphPad Prism software 4.0 (GraphPad Software, San Diego, CA, USA). All data are reported as mean±standard deviation (SD). Statistical comparisons of the groups were performed by non-parametric Kruskal-Wallis one-way analysis of variance followed by Dunn’s posttest or Mann-Whitney test. Probability value \( p<0.05 \) was considered to be statistically significant.

Results

ArtinM reduced the number of preneoplastic foci. Lesions were identified as preneoplastic foci, more specifically, as altered hepatic foci (AHF) and quantified by histological analysis using H&E staining. Our data revealed a significant increase in AHF in all of the animals exposed to DEN (0.33±0.21) compared to the control group (Ct) which did not show significant foci (\( p=0.0008 \)). Treatment with ArtinM reduced the AHF number in the D+AM animal group (6.83±1.23) compared to control animals D (15.58±2.55) (\( p=0.02 \)) (Figure 1).

ArtinM diminished cell proliferation and increased apoptosis in hepatocytes. The PCNA-labeled hepatocytes were more frequently observed in all of the DEN exposed groups compared to the corresponding control group (lack of focus) (0.16±0.16) (\( p=0.0008 \)). The number of PCNA-positive cells was reduced in D+AM group (11.50±1.63) compared to the D group (22.42±4.27) (\( p=0.01 \)) (Figure 1). Due the early period of carcinogenesis TUNEL staining showed scant nuclear staining in Ct group (0.88±0.05), that was significantly increased in D (1.37±0.06) and D+AM (2.05±0.12) groups (\( p=0.008 \) and \( p=0.008 \)). In addition, there was a significant increase of TUNEL labeled cells in the D+AM group compared to the D group (\( p=0.006 \)) (Figure 1).

ArtinM increased nuclear staining while reduced cytoplasmic staining for p21 and p27. A greater number of p21 cytoplasmic stained cells was identified in groups D (8.44±0.63) and D+AM (2.43±0.43) compared to group Ct (0.33±0.21) (\( p=0.0008 \) and \( p=0.0010 \)). However, a significant decrease of p21 cytoplasmic staining was observed in the group D+AM compared to the group D (\( p=0.003 \)). The opposite pattern was observed in the nuclear staining of p21. There was a higher number of nuclear p21 labeled cells in group D+AM (17.00±1.70) compared to group D (9.46±0.97) (\( p<0.0001 \)) (Figure 1). Similarly to p21, there was a higher number of p27 cytoplasmic stained cells in groups D and D+AM compared to group Ct (0.16±0.16) (\( p=0.0008 \)), a significant reduction in cytoplasmic p27 stained cells in group D+AM (11.33±1.22) compared to group D (17.00±1.89) (\( p=0.04 \)). Moreover, ArtinM treatment increased the nuclear p27 stained cells in group D+AM (11.24±1.57) compared to group D (4.01±0.44) (\( p=0.0006 \)) (Figure 1).

ArtinM increased p53, p42/44 MAPK, p21 and decreased cyclin D1 protein levels. The Western blot analysis revealed that group D+AM (5.27±2.36) showed an increase in p53 protein levels compared to group D (0.36±0.11) (\( p=0.03 \)). A significant increase was also identified in group D+AM...
compared to group Ct (0.58±0.02) (p=0.04), but no differences were detected between groups D and Ct (p=0.05) (Figure 2). A similar increase was detected in p42/44 MAPK protein levels of group D+AM (4.87±0.45), compared to group D (2.20±0.28) (p=0.03). However, both groups D and D+AM exhibited increased p42/44 MAPK protein levels compared to group Ct (0.48±0.15) (p=0.03 and p=0.03, respectively) (Figure 2). Finally, a statistically significant increase of p21 and decrease of cyclin D1 was identified in group D+AM (1.30±0.16 and 0.63±0.07, respectively) compared to group D (0.93±0.01 and 1.29±0.09, respectively) (p=0.03 and p=0.03, respectively). Regarding p21, there was a significant increase in groups D and D+AM compared to group Ct (0.39±0.04) (p=0.03 and p=0.03, respectively) while cyclin D1 protein levels were increased in the animals of group D compared to group Ct (0.48±0.11) (p=0.03) but in group D+AM cyclin D1 levels were similar to the levels of group Ct (p=0.48) (Figure 2).

ArtinM increased IFN-γ and TNF-α mRNA expression. Expression analysis of key inflammatory cytokine transcripts showed a significant increase of IFN-γ and TNF-α mRNA
levels in group D+AM (2.03±0.23 and 2.83±0.17, respectively) compared to group D (1.41±0.08 and 2.25±0.18, *p*=0.04 and *p*=0.03, respectively) (Figure 2). However there were no differences in gene expression of IL-12 between groups D (2.21±0.27) and D+AM (2.13±0.12) (*p*=0.69) (Figure 2).

**Discussion**

DEN is an extensively studied liver carcinogen as a promoting agent of preneoplastic changes (22). Our data confirm this concept because all the animals that received DEN presented preneoplastic foci, while in the control group, no hepatocyte damage was observed. Conversely, ArtinM treatment diminished the number of preneoplastic lesions in DEN-treated animals. Thus, ArtinM may function as a cancer preventing agent.

Although the importance of each molecular alteration during the hepatocarcinogenesis process is varied and not largely known, the progression of normal to preneoplastic hepatocytes is primarily dependent on an increased proliferation and survival activity of transformed cells (7). PCNA is a nuclear polypeptide chain protein that is directly involved in DNA replication. Increased PCNA expression is associated with pre-cancerous and cancerous cellular proliferation during hepatocarcinogenesis (23, 24). In accordance, this study confirmed an enhancement of cellular proliferation in group D compared to the control group,
based on PCNA expression pattern and showed that ArtinM treatment can diminish the percentage of PCNA-positive cells in the treated animals compared to group D indicating that ArtinM may abolish cellular proliferation.

Aberrations in G₁ phase checkpoint are critical in tumorigenesis and neoplastic progression. The progression to G₁ phase in mammalian cells is controlled mainly by the cyclin-dependent kinase complex (CDKC, cyclin-CDK) regulatory pathway. CDKC inhibitors are independently affected and aberrant expression of one or more inhibitors contributes to the carcinogenesis in the majority of HCCs cases (25). It has been reported that the maintenance of p21 and p27 proteins in the nucleus may inhibit the cyclin/CDK complex. However the cytoplasmic displacement of p21 and p27 result in loss of their activity and may contribute to cellular proliferation (26). Moreover, elevated expression of cytoplasmic p21 contributes to the development and progression of HCC (27). Similarly, the cytoplasmic sequestration of p27 is associated with cell proliferation in hepatocarcinogenesis (28). Our results corroborate the above studies by showing that animals exposed to DEN show an increase of p21 and p27 cytoplasmic staining compared to control group. Importantly, ArtinM treatment allowed a drastic reduction in the number of cytoplasmic positive cells for both p21 and p27 proteins compared to animals that received only DEN. The opposite staining pattern was observed in the nuclear localization of p21 and p27. ArtinM treatment increased nuclear p21 and p27 staining which could restore their tumor-suppressing function. Moreover, previous studies reported the importance of cyclin D-CDK complex for cell cycle transition and the intrinsic relationship between p21 levels and cyclin D1 activity (29, 30). In accordance, animals exposed to DEN, showed a significant increase of cyclin D levels, which were restored to same levels as in the control group by ArtinM treatment.

P53 is crucial tumor-suppressor gene in HCC and p53 inactivation is an important step during development of the DEN-induced HCC model (31). P53 prevents carcinogenesis by inducing cell-cycle arrest or apoptosis (32). In addition, the reduced response of p53 to DNA damage during preneoplastic progression may confer growth advantages to affected cells (33). In the present study, p53 protein show basal expression in DEN-induced animals compared to control and increased expression by ArtinM stimulation. Thus, ArtinM may re-establish the p53 levels to facilitate tumor suppression.

MAPK is involved in many cellular responses such as proliferation, differentiation, motility, and apoptosis (34). Several studies on MAPK activation in HCC (35, 36, 37) indicate that hepatocytes expressing p42/44 MAPK may represent pre-malignant cancer cells (38). Furthermore, the activation of MAPK-dependent pathways increase p53 levels and the downstream responses related to p53 induction, such as apoptosis and cell cycle arrest (39). Additionally, MAPK-p53 stimulation was associated with apoptosis and cell cycle arrest in mice with HCC (40). P42/44 MAPK activity may also be involved in the control of cellular proliferation. Induction of p21 occurs most notably by the p53 pathway (41) but there are studies reporting that activation of p21 may occur independently via p42/44 MAPK activation (42). In this regard, ArtinM treatment could increase p42/44 MAPK levels in animals that had received DEN, restored p53 levels and induced cell cycle arrest via p21 stimulation and preneoplastic hepatocyte apoptosis, as observed by TUNEL assay analysis. The nuclear translocation of p21 may be responsible for the diminished cyclin D1 levels and reduced cellular proliferation.

Dysregulation of extracellular signals like growth factors and cytokines and their receptors/downstream signaling pathways is considered as major tumorigenic events. TNFα mediates various biological effects, including cell proliferation and apoptosis, and has an important role in hepatic carcinogenesis (43). In agreement, DEN-treated animals showed an increase in TNFα mRNA expression compared to control group. Moreover, ArtinM treatment triggered a higher TNFα mRNA expression in animals exposed to DEN compared to animals exposed to just DEN. This finding is in accordance to previous studies, which demonstrated that ArtinM induces the TNFα pathway (13, 17, 44). Since TNFα is involved in apoptosis during hepatocarcinogenesis (43), induced TNFα mRNA expression by ArtinM may explain the increased number of apoptotic preneoplastic hepatocytes observed in the present study.

IFNγ is an important cytokine regulating the carcinogenesis process. Previous studies have reported that lymphocytes and natural killer (NK) cells can produce IFNγ with anti-tumor activity (45). Although ArtinM had previously been demonstrated in murine models of infection to stimulate IFNγ expression (13, 15, 17), the present study is the first to detect a significant increase in mRNA expression of IFNγ in response to ArtinM treatment during hepatocarcinogenesis. Increased IFNγ expression was accompanied by a reduction of preneoplastic foci. Furthermore, HCC is a condition closely related to human viral hepatitis, such as hepatitis B or C and since IFNγ is a cytokine that has representative anti-viral activity by regulation of replication, (46), the stimulation of IFNγ by ArtinM may provide additional advantages for this lectin in preventing HCC. Finally, IFNγ can induce cell cycle arrest via the p21-regulated signal transducers and activators of transcription (STAT) (47).

ArtinM is also associated in the stimulation of IL12 production (14). However, in this study no effect was observed in the IL12 mRNA expression by ArtinM treatment. The absence of ArtinM effect in IL12 expression in our findings may be related to the transitory release of IL12 that
may not be possible to detect to the lengthy model of hepatocyte injury employed.

In conclusion, our results established that ArtinM stimulated the production of TNFα and IFNγ cytokines. ArtinM also increased p53 levels which were accompanied by variation of p42/44 MAPK and p21 activity. These molecular changes could be responsible for the inhibition of cell-cycle progression and induction of apoptosis indicating that ArtinM may provide protection against hepatocarcinogenesis as a result of induction of cell cycle blockage and pro-apoptotic mechanisms.

Acknowledgements

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