Anti-neoplastic action of aspirin against a T-cell lymphoma involves an alteration in the tumour microenvironment and regulation of tumour cell survival

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Synopsis

The present study explores the potential of the anti-neoplastic action of aspirin in a transplantable murine tumour model of a spontaneously originated T-cell lymphoma designated as Dalton’s lymphoma. The antitumour action of aspirin administered to tumour-bearing mice through oral and/or intraperitoneal (intratumoral) routes was measured via estimation of survival of tumour-bearing mice, tumour cell viability, tumour progression and changes in the tumour microenvironment. Intratumoral administration of aspirin examined to assess its therapeutic potential resulted in retardation of tumour progression in tumour-bearing mice. Oral administration of aspirin to mice as a prophylactic measure prior to tumour transplantation further primed the anti-neoplastic action of aspirin administered at the tumour site. The anti-neoplastic action of aspirin was associated with a decline in tumour cell survival, augmented induction of apoptosis and nuclear shrinkage. Tumour cells of aspirin-treated mice were found arrested in G0/G1 phase of the cell cycle and showed nuclear localization of cyclin B1. Intratumoral administration of aspirin was accompanied by alterations in the biophysical, biochemical and immunological composition of the tumour microenvironment with respect to pH, level of dissolved O2, glucose, lactate, nitric oxide, IFN-γ (interferon-γ), IL-4 (interleukin-4), IL-6 and IL-10, whereas the TGF-β (tumour growth factor-β) level was unaltered. Tumour cells obtained from aspirin-treated tumour-bearing mice demonstrated an altered expression of pH regulators monocarboxylate transporter-1 and V-ATPase along with alteration in the level of cell survival regulatory molecules such as survivin, vascular endothelial growth factor, heat-shock protein 70, glucose transporter-1, SOCS-5 (suppressor of cytokine signalling-5), HIF-1α (hypoxia-inducible factor-1α) and PUMA (p53 up-regulated modulator of apoptosis). The study demonstrates a possible indirect involvement of the tumour microenvironment in addition to a direct but limited anti-neoplastic action of aspirin in the retardation of tumour growth.

Key words: apoptosis, aspirin, Dalton’s lymphoma, tumour microenvironment, T-cell lymphoma

INTRODUCTION

Several non-steroidal anti-inflammatory drugs routinely recommended for the prevention of cardiovascular and others ailments are being tested for the development of anti-neoplastic therapeutic strategies [1–4]. Aspirin has been being investigated for its anti-neoplastic action against a variety of malignancies and tumour cell lines including those of gastric, colorectal, prostate, pancreatic, oesophageal, breast, cervical, lymphoid and ovarian origin [5–10]. Although the precise mechanisms of the anti-neoplastic action of aspirin remain elusive, it has been reported to involve the induction of apoptosis in tumour cells by regulating cyclo-oxygenase-dependent and -independent pathways [11,12], inhibition of NF-κB (nuclear factor κB) [13,14] and release of cytochrome c from mitochondria after caspase activation [8]. Studies also report the involvement of p53, Bcl-2 and Mcl-1 in aspirin-induced apoptosis in transformed cells [13]. However,
the question of what are the possible indirect mechanism(s) of the anti-neoplastic activity of aspirin that may strongly influence the growth and survival of the tumour cells through alteration in crucial parameters of the tumour microenvironment, such as those related to alteration of intratumoral pH, glucose transport and cytokine composition, remains unexplored.

Considering the fact that T-cell malignancies are one of the most complicated ones for treatment and clinical management and have a high mortality rate [15], it is essential to understand host–tumour interactions in such malignancies for designing effective therapeutic strategies. Murine tumour models of spontaneous origin have been considered to mimic human malignancies closely [16], and therefore using a transplantable murine tumour model of a T-cell lymphoma of spontaneous origin, designated as DL (Dalton’s lymphoma), we have carried out a wide variety of investigations to delineate the host–tumour relationship. Our earlier studies have shown that tumour progression is largely dependent on factors contained in the tumour microenvironment that regulate tumour growth [17–22]. Interestingly, aspirin has been shown to induce apoptosis in various B- and T-cell lymphoma cell lines in vitro [6,13].

However, its in vivo effects on tumour progression of such malignancies in general, and on parameters of the tumour microenvironment in particular, remain unclear.

In view of these observations, the present study was carried out to investigate some of the so far unexplored aspects of the anti-neoplastic action of aspirin with the following questions regarding its mechanisms of action. (i) Does aspirin has any therapeutic efficacy against a T-cell lymphoma in vivo and can it be further optimized depending on the route of administration? (ii) Is the anti-neoplastic action of aspirin associated with alteration in biophysical, biochemical and immunological composition of the tumour microenvironment? (iii) Does the antitumour activity of aspirin involve modulation of the expression of critical cell survival regulatory proteins and genes? To address these issues tumour-bearing mice were treated with aspirin orally and/or intraperitonealnally (intratumoral) in indicated groups to mimic prophylactic and therapeutic regimens experimentally and study the overall impact of the same on tumour progression, tumour cell survival and various parameters of the tumour microenvironment.

MATERIALS AND METHODS

Mice and the tumour system
Pathogen-free inbred adult male mice of Balb/c (H-2d) strain were used at 8–12 weeks of age. The mice received food and water ad libitum and were treated with utmost humane care in an ethically certified animal room facility at the School of Biotechnology, Banaras Hindu University with approval of the institutional ethical committee for the same. DL cells were maintained in ascitic form by serial transplantation in mice (1 × 10^5 per mouse, intraperitoneal, in 0.2 ml of PBS) or in an in vitro cell culture system by serial passage as described previously [21].

Reagents
The tissue culture medium RPMI 1640 and fetal calf serum were purchased from Hyclone and the reagents, if not specified, were procured from the Sigma–Aldrich. Antibodies against IFNγ (interferon γ), IL-4 (interleukin-4), IL-6, IL-10, TGF-β (transforming growth factor-β), Hsp70 (heat-shock protein 70), V-ATPase (vacuolar proton pump), MCT-1 (monocarboxylate transporter-1) and GLUT1 (glucose transporter-1) were purchased from Chemicon and survivin, SOCS-5 (suppressor of cytokine signalling-5), cyclin B1 and VEGF (vascular endothelial growth factor) were from Imgenex. Secondary antibodies conjugated with alkaline phosphatase were obtained from Bangalore Genie. BCIP (5-bromo-4-chloroindol-3-γl phosphate)/NBT (Nitro Blue Tetrazolium) was purchased from Amresco. The Annexin V–FITC Apoptosis Detection kit was purchased from BD Pharmingen. Primers for various genes were obtained from Hysel. All the cell cultures were carried out at 37°C in a CO2 incubator (Sheldon) having 5% CO2 in air in a humidified atmosphere in a culture medium supplemented with 10% (v/v) fetal calf serum, 20 μg/ml gentamicin, 100 μg/ml streptomycin, 100 IU (international units) of penicillin, which was purchased from HiMedia. All the reagents used in the experiments were endotoxin-free. Aspirin was a gift from Alta Laboratory.

In vivo administration of aspirin
Mice were transplanted with DL (1 × 10^5 cells per mouse in 0.2 ml of PBS, intraperitoneal) in groups of ten each. Tumour cells and DLAF (DL ascitic fluid) were harvested from tumour-bearing mice on the indicated days after the tumour transplantation as described previously [21]. Aspirin was administered to tumour-bearing mice before or after tumour transplantation either orally (100 mg/kg of body weight) or by intraperitoneal injection (20 mg/kg of body weight) as per the protocol described in Figure 1. This dosage is equivalent to a daily human dosage of 80–110 mg/day [23,24].

Monitoring the tumour progression and survival of tumour-bearing mice
Tumour growth was monitored by measuring the change of body weight, estimation of viable tumour cell density in DLAF by a standard Trypan Blue dye exclusion test and survival of tumour-bearing mice by following a method described previously [22].

Preparation of cell-free DLAF
DLAF was prepared by a method described previously [20]. Tumour cells were aspirated by peritoneal lavage on indicated days after tumour transplantation. The cell suspension was centrifuged at 200 g for 10 min. The supernatant was collected and its volume was measured; it was used after passing through a 0.22 μm membrane filter. Protein content was measured by the standard Bradford assay (Bio-Rad) using in-house BSA standards.
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Figure 1  Study design of aspirin administration before or after tumour transplantation in tumour-bearing mice (Balb/c mice)

Tumour-bearing mice (10 mice per group) were administered either orally or intraperitoneally with aspirin or PBS before and after intraperitoneal injection of $5 \times 10^5$ tumour (DL) cells in 0.2 ml of PBS. Days of tumour transplantation was taken as the time of reference (day 0).

Assay for tumour cell survival by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide]

Freshly collected or in vitro cultured tumour cells seeded ($1 \times 10^5$ viable cells; 100 µl per well) in a 96-well tissue culture plate were incubated with 50 µl of an MTT solution (5.0 mg/ml in PBS) at 37°C for 4 h. The plate was then centrifuged at 100 g for 5 min at 4°C. After removal of supernatant, the cell pellet was dissolved in 50 µl of DMSO to solubilize the formazan crystals formed. The plates were read on a microplate reader (Labsystems) at a wavelength of 540 nm. Readings are presented as percentage cell viability, calculated with reference to tumour cells from control mice or tumour cells incubated in vitro in the absence of aspirin.

Flow cytometric analyses of apoptotic cell death and cell cycle

Tumour cells harvested from tumour-bearing mice were washed with cold PBS and $1 \times 10^5$ cells were stained either with a Becton Dickinson Annexin V–FITC and PI (propidium iodide) kit as per the manufacturer’s protocol or with PI (10 µg/ml) alone after incubation with 20 µg/ml RNase A for 30 min at 37°C in the dark. A total of 10 000 events were acquired on a flow cytometer (FACS Aria; Becton Dickinson) and analysed by FlowJo software (Tree Star).

Nuclear size estimation

Morphometric analysis to determine nuclear morphology and size was carried out by a method described previously [25]. Tumour cells ($0.5 \times 10^5$), adhered on poly-L-lysine-coated glass slides by Cytospin (ThermoScientific), were fixed with Carnoy’s fixative (methanol/acetic acid; 3:1, v/v), permeabilized in saponin (10 min) and stained with DAPI ($4',6$-diamidino-2-phenylindole; 1 µg/ml) for 10 min at room temperature (25°C) in the dark and finally mounted with fluoromount antifade agent. Fluorescence images were captured using an Olympus fluorescence microscope at $\times 100$ magnification. Nuclear size estimation was performed using ImagePro software.
Confocal microscopy for immunodetection of subcellular localization of cyclin B1

Tumour cells, adhered on to poly-L-lysine-coated glass slides by Cytospin using a method described previously [26], were fixed in 2 % (w/v) paraformaldehyde in PBS, rinsed in 50 mM glycine in PBS, blocked in 10 % (v/v) goat serum, incubated with an Alexa Fluor® 594-conjugated cyclin B1 antibody (1:500) for 30 min in the dark and counter-stained with DAPI (1 μg/ml). All fluorescence images were captured by a TCS SP5 confocal microscope (Leica).

Quantitative determination of apoptotic cells

Induction of apoptosis in tumour cells was measured by means of a quantitative determination of DNA fragmentation following a method routinely followed in the laboratory or TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) staining using a TUNEL assay kit (Invitrogen), as described previously [21]. The percentage of DNA fragmentation was calculated as follows:

\[
\text{DNA fragmentation (\%: normalized to untreated control)} = \frac{F}{F + B} \times 100
\]

where \( F \) is the absorbance of fragmented DNA and \( F + B \) is the absorbance of total DNA.

Nitrite assay

The concentration of stable nitrite, the end-product from NO generation and an indicator of macrophage activity, was determined in the DLAF by the methods described previously [17] based on the Griess reaction. DLAF was incubated with an equal volume of Griess reagent [1 part of 1 % (w/v) sulfanilamide in 2.5 % H₃PO₄ plus 1 part of 0.1 % naphthyl-ethylene-diamine dihydrochloride; the two parts being mixed together within 12 h of use and kept chilled] at room temperature for 10 min in a 96-well microtitre plate. The absorbance at 540 nm was determined with an automatic ELISA plate reader. Nitrite content was quantified by extrapolation from a standard curve of NaN₃ in each experiment. In all the experiments, nitrite content in the wells containing medium without cells was also measured and subtracted.

Quantification of dissolved oxygen

DLAF samples were analysed for oxygen content immediately after harvest by using a method described previously [18]. In brief, an aliquot of the sample was treated with MnSO₄ and a strong alkaline iodide reagent (10 g/l NaN₃, 135 g/l NaI and 500 g/l NaOH). The Mn(OH)₂ formed reacts with the dissolved oxygen to form a brown precipitate of MnO(OH)₂. On acidification, in the presence of iodide, the iodine liberated was equivalent to the dissolved oxygen originally present in the sample. The iodine was titrated with a standardised Na₂S₂O₃ solution; starch was used as an indicator. Oxygen content was expressed in terms of p.p.m.

Quantification of glucose

Glucose content in the DLAF was measured using a commercial kit from Agappe Diagnostics based on conversion of glucose into H₂O₂ by the action of glucose oxidase. Final estimation of the generated H₂O₂ was performed by converting it into a red quinone product through peroxidase action [18]. Briefly, 10 μl of tumour ascitic fluid was mixed with 1 ml of working reagent containing sodium phosphate buffer (pH 7.4), phenol, glucose-oxidase, peroxidase and 4-aminophenylpyrine and was incubated for 10 min at 37 °C. The readings were taken at 505 nm. Glucose content is expressed in terms of mg/dl.

Estimation of lactate

Lactate concentration in DLAF was measured using an enzymatic colorimetric method (Spinreact) [27]. Briefly, 1 μl of the sample was diluted in 200 μl of 50 mM Pipes (pH 7.5) containing 4-chlorophenol (4 mM), lactate oxidase (800 units/l), peroxidase (2000 units/l) and 4-aminophenazole (0.4 mM), followed by incubation for 10 min at room temperature, and measurement of absorbance at 505 nm. Lactate concentration was expressed in terms of mg/dl.

Immunoblot analysis

Expression of various proteins in tumour cells was checked by using a method described previously [22]. Briefly, 30 μg of Triton X-100 solubilized proteins isolated from whole tumour cell lysates, cytoplasmic or nuclear extracts, prepared by a method described by Bharti et al. [28], were resolved on an SDS/10 % polyacrylamide slab gel. The separated proteins were transferred onto a nitrocellulose membrane (Sartorius), immunoblotted with different antibodies followed by probing with a corresponding secondary antibody conjugated with alkaline phosphatase and detected by a BCIP/NBT solution. The images of immunoblots thus obtained were captured on a gel documentation image analysis system (Bio-Rad) and the intensity of bands was analysed by using QuantityOne software (Bio-Rad). β-Actin was used as an internal control.

RT–PCR (reverse transcription–PCR) analysis

RT–PCR analysis for the expression of mRNA of HIF-1α (hypoxia-inducible factor-1α) and PUMA (p53 up-regulated modulator of apoptosis) transcript was conducted by a method described previously [22] with a one-step RT–PCR cell to cDNA kit (Ambion). Primer sequences and annealing temperature are given in Table 1. PCR was performed for 15 min to make cDNA at 50 °C. The amplification was carried out for 35 cycles with initial denaturation at 94 °C for 2 min, followed by annealing for the indicated duration and elongation at 72 °C for 30 s. The samples were separated on an agarose gel (1 %) containing ethidium bromide (0.3 mg/ml). The bands were visualized on a UV-transilluminator and captured by a gel doc image analysis system (Bio-Rad).

Cytokine ELISA

Standard ELISA was performed to detect the presence of indicated cytokines in DLAF following a method described
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RESULTS

Retardation of tumour progression after administration of aspirin to the tumour-bearing host

To assess the prophylactic and therapeutic anti-neoplastic action of aspirin on DL tumour cells in vivo, tumour-bearing mice were treated (Figure 1). Therapeutic or prophylactic effects of aspirin were primarily monitored through measurement of overall survival, change in body weight, viable tumour cell counts in DLAF and tumour cell viability. Administration of aspirin to tumour-bearing mice either in non-prophylactic treatment schedule group 2 and 4 or in prophylactic treatment schedule group 6 resulted in retardation of tumour progression along with augmented survival of tumour-bearing mice (Table 2). A prolonged survival of aspirin-treated mice was accompanied by a reduced change in body weight, viable tumour cell counts in ascitic fluid and lower cell viability as early as day 10 irrespective of the type of aspirin treatment regimen (Table 2). Tumour-bearing mice in groups 2, 4 and 6 had almost 60 % overall survival by day 25 as compared with 20-30 % survival in the respective control groups (group 1 and group 3; Table 2). Although these differences in survival after tumour transplantation persisted until day 35, by day 40 all the mice of non-prophylactic or prophylactic groups died. Interestingly, the group of mice treated with a combination of oral (prophylactic) and intratumoral (therapeutic) aspirin (group 7) showed maximal survival as 90 % of the mice survived till day 20 and 20 % of mice were still surviving at day 40. The differential effect with respect to change in body weight, viable tumour cells count in DLAF and tumour cell viability in aspirin-treated groups was most conspicuous by day 20 after mice in control groups started losing viability rapidly and further comparison was not feasible. In parallel with the improvement in overall survival that was observed in aspirin-treated mice of group 7, the tumour cell density in the DLAF of these mice displayed maximal decrease. When these cells were subjected to measurement of cell survival by the MTT assay, cells of aspirin-treated mice were found to be metabolically less active or losing their viability (group 2: 60 %; group 4: 67 %; and group 6: 64 %) as compared with the control group (group 1). The cytotoxic action of aspirin was optimal when it was administered in prophylactic combination of both oral and intratumoral intra-peritoneal injection (group 7). Thus administration of aspirin orally prior to tumour transplantation followed by its administration intra-peritoneally was observed to be the most effective treatment regimen and was investigated further in subsequent experiments.

<table>
<thead>
<tr>
<th><strong>Table 1</strong> Primers for RT-PCR analysis</th>
<th><strong>Annealing temperature</strong></th>
<th><strong>Reference</strong></th>
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<tr>
<td>PUMA</td>
<td>F’-CCTCAGCCTGTCACCAG-3’</td>
<td>68°C</td>
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<td></td>
<td>R’-CCGCCGCTCTGACTGCGCGTT-3’</td>
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<tr>
<td>HIF-1</td>
<td>F’-TGAGCTCACCACATTTGAATAAGCTTCT-3’</td>
<td>67°C</td>
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<tr>
<td></td>
<td>R’-GGGCTTCCAGATAAAAACAGGCTCAT-3’</td>
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<tr>
<td>β-Actin</td>
<td>F’-GGACACGTGTTGGTGAC-3’</td>
<td>54°C</td>
</tr>
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<td></td>
<td>R’-CTGGACCACACCTTCTAC-3’</td>
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Statistical analysis

All experiments were conducted in triplicate at least three times. Data analysis was performed using the statistical software SPSS version 17. The Student’s t test was used to compare the difference between test groups. The difference was considered statistically significant when P < 0.05. Results are expressed as means ± S.D.

Effect of aspirin administration on induction of tumour cell death and cell cycle progression

To determine whether aspirin mediates antitumour effects through induction of cell death and/or disturbing the progression of cell cycle in tumour cells, tumour cells harvested from tumour-bearing mice of the control (group 1) and the aspirin-treated group (group 7) were stained with annexin V–PI and analysed by flow cytometry. The dual parametric dot plot shown in Figure 2(A) demonstrates that more than 98 % of tumour cells derived from control tumour-bearing mice were viable. On the other hand, the proportion of live tumour cells was reduced to 77 % in aspirin-treated mice and a significant proportion of tumour cells was detected in different stages of apoptotic cell death. Tumour cells were also subjected to cell cycle analysis by flow cytometry in order to assess the aspirin-induced changes in cell cycle progression. As shown in Figure 2(B), 31 and 18 % of
Table 2 Effect of aspirin administration on tumour progression

Mice were treated as per the protocol mentioned in Figure 1. Ascitic fluid (0.1 ml) was harvested on day 10 and day 20 after tumour transplantation to check viable tumour cell density by Trypan Blue exclusion. Tumour progression was monitored by recording the change of body weight, survival of tumour-bearing mice and cell viability by MTT assay as described in the Materials and methods section. Values shown are means ± S.D. for a representative experiment. *P < 0.05 compared with values for respective controls. †P < 0.05 compared with values for group 2. ‡P < 0.05 compared with values for groups 1 and 3. §P < 0.05 compared with values for groups 4 and 5. ||P < 0.05 compared with values for groups 2, 4 and 6.

<table>
<thead>
<tr>
<th>Parameters of tumour progression</th>
<th>Days after tumour transplantation</th>
<th>Percentage change in body weight after tumour transplantation (×10⁶/ml of ascitic fluid)</th>
<th>Viable tumour cells (×10⁶/ml ascitic fluid)</th>
<th>Percentage cell viability of tumour cells (by MTT assay)</th>
<th>Percentage survival of tumour-bearing mice</th>
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<tr>
<td></td>
<td>10</td>
<td>20</td>
<td>20</td>
<td>20</td>
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<td>Therapeutic (treatment after tumour transplantation)</td>
<td>PBS (group 1)</td>
<td>Aspirin (group 2)</td>
<td>Water (group 3)</td>
<td>Aspirin (group 4)</td>
<td>Control (group 5)</td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td>54.7 ± 3.7</td>
<td>39.9 ± 1.1*</td>
<td>59.9 ± 3.1</td>
<td>49.9 ± 3.0†</td>
<td>45.7 ± 1.3†</td>
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<td>Oral</td>
<td>67.1 ± 2.1</td>
<td>41.9 ± 0.3*</td>
<td>69.9 ± 4.1</td>
<td>46.3 ± 1.1*†</td>
<td>56.3 ± 0.7†</td>
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<td>Prophylactic (treatment before and after tumour transplantation)</td>
<td>Control (group 5)</td>
<td>Oral only (group 6)</td>
<td>Oral and intraperitoneal (group 7)</td>
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<td>Therapeutic (treatment after tumour transplantation)</td>
<td>4.8 ± 0.03</td>
<td>6.6 ± 0.31*</td>
<td>95 ± 3.7</td>
<td>62 ± 2.1*†</td>
<td>85 ± 2.9†</td>
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<td>Intraperitoneal</td>
<td>9.6 ± 0.01</td>
<td>6.6 ± 0.31*</td>
<td>8.8 ± 0.34</td>
<td>7.21 ± 0.07†</td>
<td>6.31 ± 0.82†</td>
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<tr>
<td>Oral</td>
<td>100 ± 3.2</td>
<td>53 ± 2.6*</td>
<td>95 ± 3.7</td>
<td>62 ± 2.1*†</td>
<td>89 ± 2.7†</td>
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<tr>
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<td>Oral only (group 6)</td>
<td>Oral and intraperitoneal (group 7)</td>
<td></td>
<td></td>
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<tr>
<td>Percentage change in body weight after tumour transplantation (×10⁶/ml of ascitic fluid)</td>
<td>PBS (group 1)</td>
<td>Aspirin (group 2)</td>
<td>Water (group 3)</td>
<td>Aspirin (group 4)</td>
<td>Control (group 5)</td>
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<td>Therapeutic (treatment after tumour transplantation)</td>
<td>Therapeutic (treatment after tumour transplantation)</td>
<td>PBS (group 1)</td>
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<td>Water (group 3)</td>
<td>Aspirin (group 4)</td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td>25</td>
<td>20 ± 7.9</td>
<td>60 ± 8.7</td>
<td>30 ± 7.1</td>
<td>60 ± 12.7</td>
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<tr>
<td>Oral</td>
<td>30</td>
<td>0</td>
<td>30 ± 5.7*</td>
<td>0</td>
<td>30 ± 16*†</td>
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<tr>
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<td>Oral only (group 6)</td>
<td>Oral and intraperitoneal (group 7)</td>
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</table>
| Percentage change in respective body weight of each mouse with respect to its initial body weight at the time of tumour transplantation.

*P < 0.05 compared with values for respective controls. †P < 0.05 compared with values for group 2. ‡P < 0.05 compared with values for groups 1 and 3. §P < 0.05 compared with values for groups 4 and 5. ||P < 0.05 compared with values for groups 2, 4 and 6.
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Figure 2  Effect of aspirin administration on tumour cell survival and cell cycle progression in tumour-bearing mice

(A) In vivo induction of apoptosis in tumour cells harvested from aspirin-administered mice. Flow cytometric evaluation of tumour cells harvested on day 15 after tumour transplantation from tumour-bearing mice of control (Group 1) and aspirin-treated (Group 7) groups stained with annexin V-FITC and PI. Dot plots showing cells in different phases of apoptotic cell death. Cells in quadrant Q1 represent necrotic cells, Q2 late apoptotic cells, Q3 live cells and Q4 early apoptotic cells. Values shown represent the percentage of cells in each quadrant. (B) Tumour cells of aspirin-treated mice get arrested at G1 phase of cell cycle. Dot plots showing forward scatter (FSC) and side scatter (SSC) profiles (upper panels) and histograms depicting PI-stained tumour cells of control and aspirin-treated mice in different phases of the cell cycle (lower panels). (C, D) Aspirin-treated tumour cells show nuclear shrinkage. Representative forward scatter, PI-W and PI-A histograms of DNA content captured through PI staining of control (white bars) and aspirin-treated cells (black bars), showing the shifting of peaks to the left, which is indicative of shrinkage of PI-stained nuclei (C). Fluorescence images of the respective nuclei by DAPI staining (D). Nuclear area of DAPI-positive tumour cells was measured as described in the Materials and methods section, and cumulative data are presented as arbitrary units (E). (F–H) Nuclear localization of cyclin B1. Immunofluorescence photomicrograph showing cyclin B1 localization in tumour cells of control and aspirin-treated mice. Cells were counter-stained with DAPI to visualize the nucleus (F). Altered levels of cyclin B1 in cytoplasmic and nuclear fractions of tumour cells exposed to aspirin (G). Cytoplasmic and nuclear fractions were processed for Western blotting of cyclin B1 as described in the Materials and methods section. Relative intensity of cyclin B1 with respect to β-actin was determined by densitometric evaluation (H).

Tumour cells were in S and G2/M phases, respectively, in tumour cells derived from control mice (group 1), whereas the proportion of these dividing cells decreased in the aspirin-treated group (group 7; S phase: 11%; G2/M phase: 6%). Interestingly, tumour cells from aspirin-treated mice showed an increase in cells with sub-G1 DNA content, which is a hallmark feature of cells undergoing apoptotic cell death. Further, a shift in the FSC-A, PI-W and PI-A was observed in tumour cells of aspirin-administered
tumour-bearing mice, indicating that tumour cells from aspirin-administered mice displayed cell and nuclear shrinkage (Figure 2C, FSC-A, PI-W and PI-A). These observations were further reconfirmed by measurement of nuclear area using fluorescence microscopy of DAPI-stained cells (Figures 2C–2E). Tumour cells were subsequently subjected to immunocytochemical analysis to assess the expression of cyclin B1 by confocal microscopy and Western-blot analysis. Results shown in Figures 2(F)–2(H) indicate the presence of cytoplasmic and nuclear cyclin B1 in tumour cells harvested from control mice; on the other hand, cyclin B1 was completely lacking in cytoplasm of aspirin-exposed tumour cells and it was located exclusively in the nuclei. The levels of cyclin B1 were also checked in the cytoplasmic and nuclear fractions of control and aspirin-exposed tumour cells. The level of cyclin B1 was found to decrease in the cytosolic fraction, accompanied by its increase in nuclear fraction in tumour cells exposed to aspirin in vivo.

Effect of aspirin administration on the composition of the tumour microenvironment

To determine the effect of aspirin administration on the tumour microenvironment which plays a pivotal role in tumour progression, we assessed DLAF obtained from tumour-bearing mice of control (group 1) and aspirin-treated groups (group 7) for biochemical (volume, dissolved O2 and pH), biochemical (glucose, nitrite and lactate) and immunological (Th1 and Th2 cytokines) constitution. Aspirin-treated mice had lower volume of ascitic fluid but higher levels of dissolved O2 and had neutral pH as compared with DLAF from control mice, which was slightly acidic and had a comparatively lower O2 content (Table 3). Similarly, the biochemical composition was also altered in aspirin-treated mice as DLAF obtained from these mice had higher glucose and nitrite contents, whereas overall lactate levels were lower compared with DLAF from control mice (Table 3). Moreover, an increase in the level of IFNγ accompanied by a decline in the levels of IL-6, IL-4 and IL-10 was observed in the DLAF of aspirin-administered tumour-bearing mice compared with control. On the other hand, TGF-β levels remained unaltered in the aspirin-exposed group compared with control (Table 3).

Effect of aspirin administration on the expression of cell survival regulatory proteins and genes

To assess the mechanism of antitumour action of aspirin on the T-cell lymphoma, tumour cells obtained from control and aspirin-treated groups were examined for expressions of GLUT1, pH regulators (MCT-1 and V-ATPase), and a panel of cell survival regulatory proteins (survivin, Hsp70, SOCS-5 and VEGF) and genes (HIF-1α and PUMA). The results shown in Figure 3 demonstrate that aspirin administration to tumour-bearing mice resulted in a decline in the expressions of survivin, GLUT1, Hsp70, SOCS-5, VEGF, MCT-1 and V-ATPase proteins (Figures 3A and 3B) and HIF-1 gene compared with their expression in tumour cells of control mice (Figures 3C and 3D). The inhibitory effect was most pronounced with respect to expression of the pH regulators MCT-1 and V-ATPase. In contrast, aspirin administration resulted in increased expression of PUMA transcripts in aspirin-treated tumour cells (Figures 3C and 3D).

In vitro exposure of aspirin causes a dose-dependent loss of cell viability and induction of apoptotic cell death in tumour cells

To estimate the direct action of aspirin on DL cell survival, tumour cells (1×10^5 cells per well) were incubated in vitro in media without or with the indicated doses of aspirin for 48 h followed by estimation of cell viability by the MTT assay and estimation of apoptosis by TUNEL staining and DNA fragmentation. As shown in Figure 4(A), cells treated with aspirin in vitro showed a dose-dependent loss of cell viability of cultures over the concentration range of 1–10 mM. However, treatment of tumour cells with aspirin at doses of less than 1 mM did not significantly alter cell viability compared with the control. On the other hand, doses of more than 10 mM of aspirin did not augment its cytotoxic action on tumour cells. Aspirin-treated tumour cells were also quantified for induction of apoptosis by TUNEL assay and DNA fragmentation. Tumour cells analysed under a fluorescence microscope by TUNEL assay showed increased cell death as reflected by increased fluorescence in samples treated with aspirin in a dose-dependent manner compared with untreated tumour cells (Figure 4B). These findings were supported by those from aspirin-treated cells subjected to DNA fragmentation assay, which confirmed the dose-dependent increase in fragmented DNA in cells treated with aspirin (Figure 4C). In order to correlate in vitro observations regarding the antitumour action of aspirin to those made following in vivo administration of aspirin to tumour-bearing mice about the regulation of tumour cell survival, we checked the effects of aspirin treatment in vitro of tumour cells on the expression of Hsp70 and V-ATPase proteins in a dose– and time–response manner. Results are shown in Figures 4(D)–4(G). Aspirin treatment of tumour cells caused inhibition of the expression of Hsp70 and V-ATPase depending on the dose and time of treatment.

DISCUSSION

Despite the documented evidence for the anti-neoplastic action of aspirin [5], its chemo-preventive and therapeutic benefits are debatable [29], owing to ill-defined mechanism(s) of action, in a tumour-specific manner, and an incomplete knowledge of the appropriate dosage regimen. Therefore the present study was conducted to address some of these crucial issues with respect to the mechanisms of the anti-neoplastic action of aspirin. The results of this study demonstrate an optimal therapeutic efficacy of this drug against a T-cell lymphoma, which was achieved when it was administered directly in the tumour microenvironment, compared with the conventional oral route of administration. Moreover, when administered prior to tumour transplantation, as a prophylactic strategy, aspirin was observed to prime the
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Table 3 Effect of aspirin administration to tumour-bearing mice on parameters of the tumour microenvironment

DLAF obtained from control (group 1) and aspirin-treated (group 7) tumour-bearing mice was examined for indicated physical, biochemical and immunological parameters on day 15 after tumour transplantation as described in the Materials and methods section. Values shown are means ± S.D. for a representative experiment.

<table>
<thead>
<tr>
<th>Parameter of tumour microenvironment</th>
<th>Treatment (ten mice per group)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS (group 1)</td>
<td>Aspirin-treated (group 7)</td>
</tr>
<tr>
<td>Physical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume of ascitic fluid (ml)</td>
<td>6.2 ± 1.4</td>
<td>4.6 ± 0.7*</td>
</tr>
<tr>
<td>Dissolved O₂ (p.p.m.)</td>
<td>2.8 ± 0.4</td>
<td>7.9 ± 1.0*</td>
</tr>
<tr>
<td>pH</td>
<td>6.7 ± 0.1</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td>Biochemical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>8.7 ± 2.6</td>
<td>10.2 ± 1.8</td>
</tr>
<tr>
<td>Nitrite (μM)</td>
<td>541.2 ± 23.1</td>
<td>673.8 ± 19.2*</td>
</tr>
<tr>
<td>Lactate (mg/dl)</td>
<td>33.9 ± 3.4</td>
<td>22.8 ± 4.4*</td>
</tr>
<tr>
<td>Immunological</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Th1 cytokines (A405)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1.02 ± 0.22</td>
<td>1.7 ± 0.06*</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.44 ± 0.1</td>
<td>0.34 ± 0.01</td>
</tr>
<tr>
<td>Th2 cytokines (A405)</td>
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<td></td>
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<tr>
<td>IL-4</td>
<td>0.56 ± 0.07</td>
<td>0.35 ± 0.02*</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.84 ± 0.1</td>
<td>1.38 ± 0.07*</td>
</tr>
<tr>
<td>TGF-β</td>
<td>0.29 ± 0.08</td>
<td>0.27 ± 0.07</td>
</tr>
</tbody>
</table>

Figure 3  Altered expressions of cell survival and tumour microenvironment regulatory proteins and genes in tumour cells exposed to aspirin in vivo

Representative immunoblots of cellular proteins isolated from tumour cells of control (Group-1) and aspirin-treated (Group-7) mice showing the expressions of indicated proteins (A, B). RT–PCR analysis of HIF-1α and PUMA mRNA expression (C, D). The relative intensity of respective protein/mRNA expression with respect to β-actin was determined by densitometric evaluation (B, D).
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Figure 4  Effect of aspirin on tumour cell survival, induction of apoptosis and expression of survival regulatory proteins in vitro
Tumour cells (1 × 10^5 cells per well) were incubated in medium without or with indicated concentrations of aspirin for 48 h followed by the estimation of percentage cell viability by MTT assay (A) and induction of apoptosis by percentage of DNA fragmentation (B) and TUNEL staining (C). Values shown are the means ± S.D. for a representative experiment of three independent experiments. *P < 0.05 compared with values for tumour cells incubated in a medium without aspirin. Western-blot analysis of tumour cells treated with medium without or with aspirin for the indicated dose (for 48 h) (D, E) and time (at 10 mM aspirin) (F, G) for the expression of Hsp70 and V-ATPase was carried out. Relative intensity of bands with respect to β-actin was determined by densitometric evaluation (E, G).

antitumour action of intratumoral aspirin administration. The reason for such priming action of aspirin is not known. However, it is anticipated that the same could be dependent on an altered balance of tumour survival regulatory molecules. Indeed, oral administration of aspirin in normal individuals for pain relief and cardiovascular therapies has been reported to be associated with alterations in the balance of a number of regulatory molecules such as NO and cytokines [24,30].

A significant proportion of tumour cells of aspirin-treated tumour-bearing mice were apoptotic and metabolically inferior, indicating that aspirin exerted cytostatic and cytotoxic actions on tumour cells through induction of apoptosis. In view of these observations, we explored the mechanism(s) underlying such a mode of the anti-neoplastic action of aspirin following its intratumoral administration in concert with its prophylactic regimen. Flow cytometric examination of annexin V–PI-stained tumour cells from aspirin-administered mice revealed an increase in the proportion of tumour cells with apoptotic features by gross alteration in their cell cycle, resulting in a reduced fraction of cells in S, G2 and M phases. These observations suggest a potential growth arrest at G1/S transition during the cell cycle. Moreover, the possibility of cells in S/G2/M phase being more susceptible to aspirin treatment for induction of cell death cannot be ruled out. Further, analysis of flow cytometry data complemented by microscopic observations of tumour cells revealed that aspirin-treated cells were smaller in size along with shrunken nuclei. As fast dividing malignant cells display a grossly increased mean nuclear area [31] and the increase in the nuclear size is related to an increase in the nuclear contents required for replication [32], these phenotypic features observed in tumour cells of aspirin-administered mice are therefore indicative of a quiescent nuclear chromatin contributing to cytostasis [26]. Moreover, we also observed a loss of cytosolic fraction of cyclin B1 accompanied by its predominant relocation in nuclei. Cyclin B1 has been demonstrated to drive G2/M transition [33]. Previous studies suggested that nuclear accumulation of cyclin B1 is a prerequisite for induction of apoptosis. As nuclear translocation of cyclin B1 has been reported to be associated with induction of apoptosis in several studies [34–37], we wished to investigate whether the nuclear accumulation of cyclin B1 was also associated with aspirin-induced cell death of tumour cells. Moreover, studies have also indicated that nuclear accumulation of cyclin B1 when occurs in association with DNA damage ushers the induction of apoptosis instead of promoting mitosis [33]. Nuclear translocation of cyclin B1 is
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also correlated with radiation-induced apoptosis [35]. Nevertheless metabolites of aspirin have also been reported to induce DNA damage [38]. To the best of our knowledge, none of the earlier studies on aspirin have reported its cell cycle arresting activity in vivo as one of the mechanisms involved in manifestation of its antitumour actions.

The observed cytotoxic effect of aspirin in our tumour model may be a result of its direct as well as indirect actions on tumour cells. Results of our in vitro experiments support a direct cytotoxic action of aspirin on T-cell lymphoma. Nevertheless, it is likely that the intratumoral route of aspirin administration might lead to the delivery of an effective and optimal concentration of the drug in the vicinity of the tumour, which may otherwise not be achievable if the drug was administered by the oral route alone. As factors contained in the tumour microenvironment have been demonstrated to regulate tumour cell survival [18], we also investigated whether modulation of crucial parameters of the tumour microenvironment contributed to aspirin-induced tumour cell apoptosis. Indeed, the findings of the present study demonstrate that aspirin administration to the tumour-bearing host render the composition of the tumour microenvironment unfavourable for tumour cell survival with respect to the level of pH, glucose, oxygen, lactate and cytokines. Nevertheless changes in cellular and nuclear morphology that are directly related to cell survival and proliferation [39,40] are associated with functional changes in glucose uptake and metabolism [41], which in turn may affect the oxygen content and pH of the tumour microenvironment. Thus another novel finding of the present study regarding the mechanisms of the antitumour activity of aspirin is that the observed cytotoxic action of the drug could also be due to a decreased hypoxia of the tumour microenvironment.

Our results revealed a marginal decline in the cell survival regulatory proteins such as survivin, SOCS-5 and Hsp70 [42] in tumour cells of aspirin-treated mice, whereas the expressions of two important pH regulators, namely the lactate transporter MCT-1 and the proton pump V-ATPase [43], were inhibited. Aspirin treatment of tumour cells in vitro also caused inhibition of the expression of Hsp70 and V-ATPase proteins. Thus it is suggested that the inhibitory action of aspirin on tumour cell survival could be derived via its direct action on tumour cells and/or its action mediated by factors contained in the tumour microenvironment. Interestingly, ours is the first study to show the inhibitory action of aspirin on the expression of pH regulators in tumour cells, which significantly contributes towards the development of tumour acidosis promoting tumour progression [43]. Aspirin administration also resulted in reduced GLUT1 expression in tumour cells, which could have been one of the contributory factors for the modulated glucose metabolism in tumour cells. An earlier study using breast cancer cell lines has shown that aspirin can induce alteration in glucose metabolism by altering the structure of 6-phosphofructo-1-kinase, which can prevent lactate production [44]. However, information on its effects on the expression of glucose transporter(s) in tumour cells is mostly lacking. Our study shows for the first time that aspirin can also impair glucose transport in tumour cells by targeting the pivotal glucose transporter GLUT1, which in turn may prevent these cells from acquiring a more aggressive phenotype and inducing apoptosis [45]. Such alterations in glucose metabolism have also been linked to compromised tumour cell survival [45,46]. Moreover, it is likely that a reduced GLUT1 expression and a decline in the lactate transporter MCT-1, which were accompanied by high glucose content and low lactate levels observed in DLAF obtained from aspirin-treated mice, are partly responsible for the induction of apoptotic cell death in tumour cells. Although the mechanism by which aspirin inhibited the expression of MCT-1 and V-ATPase is unknown, studies show that cytokines may be involved in the modulation of expression of pH regulators on tumour cells [21,47].

Another interesting finding of the present study was with respect to the expression of the HIF-1α gene which was down-regulated in tumour cells of aspirin-treated mice. A reduction in HIF-1α transcript may be a direct effect of the altered composition of tumour physiology and microenvironment with respect to glucose metabolism and hypoxic conditions. HIF-1α plays an important role in restoring oxygen homeostasis in oxygen-deprived cells by promoting glycolysis and angiogenesis in a variety of tumour cells and has been shown to be a key regulator of cell survival through its multifaceted controlling actions on the activity and expression of Hsp70, p53, VEGF and GLUT isoforms [21,48]. Thus the observed changes in the expression of Hsp70, GLUT1 and VEGF in tumour cells exposed to aspirin could be associated with altered expression of HIF-1α. Although direct action of aspirin on HIF-1α expression is still an unexplored preposition, a recent study has demonstrated that nitric oxide can negatively suppress HIF-1α expression in aspirin-treated macrophages [49], a finding that strongly correlates with our observations indicating higher nitrite content and reduced HIF-1α transcript in aspirin-treated tumour-bearing mice.

It is also important to note that aspirin administration to tumour-bearing hosts was associated with an increase in the levels of IFNγ. As IFNγ has been shown to be a potent activator of macrophages with tumoricidal M1 phenotype [22], the same could constitute an additional indirect mode of anti-neoplastic action of aspirin. Moreover, changes in cytokine balance in the tumour microenvironment could also be associated with a decline in tumour cell survival following aspirin administration and a decreased expression of pro-survival proteins such as survivin, SOCS-5 and Hsp70. A loss of SOCS-5 expression has been proposed to induce apoptosis [21,50]. Moreover, a decline in the level of IL-10 accompanied by an elevation of IFNγ has been reported to augment induction of tumour cell death and decline of tumour-induced immunosuppression [51]. Additionally IFNγ has been shown to augment p53-dependent killing of tumour cells [52]. Augmented expression of the PUMA gene, which is a mediator of p53-induced cell death [53] in tumour cells of aspirin-administered mice, is also suggestive of a direct action of elevated IFNγ following aspirin treatment. Moreover, aspirin can antagonize prostaglandin-mediated immunosuppressive actions [54], which are known to be manifested by overproduction of IL-10, and inhibition of IFNγ in the tumour milieu [55]. However, the mechanism of aspirin-dependent up-regulation of IFNγ expression in the tumour microenvironment is an unresolved question.
Taken together, the results of the present study strongly suggest that aspirin may also exert its antitumour activity by altering the tumour microenvironment. A summary of the main observations of the present study is presented in Figure 5. Beneficial chemo-preventive and therapeutic effects observed in cross-sectional human studies may therefore be a consequence of these direct and indirect actions which cannot be monitored in an *in vitro* study. Thus the findings of the present study will be an important milestone in the ongoing quest to understand the mechanisms of action of aspirin and will help us to optimize the usage of this widely practised, most economical drug with minimal side effects for achieving the desired therapeutic benefits in a host bearing a T-cell lymphoma.

**AUTHOR CONTRIBUTION**

Anjani Kumar contributed to conceiving the study hypothesis, conducted the experiments, helped in the analysis and interpretation of the data, and contributed to the writing and preparation of the paper; Naveen Kumar Vishvakarma provided intellectual input and contributed to conceiving the study hypothesis, analysis and interpretation of the data and writing and preparation of the paper; Abhishek Tyagi contributed to the flow cytometry experiments and to the preparation of the paper; Alok Chandra Bharti and Sukh Mahendra Singh conceived and designed the study, gave overall supervision, and contributed to the analysis and interpretation of the data and the writing and preparation of the paper. All authors read and approved the final paper.

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