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TNF-α renders macrophages resistant to a range of cancer chemotherapeutic agents through NF-κB-mediated antagonism of apoptosis signalling

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ABSTRACT

The abundance of macrophages is an independent negative prognostic factor in a range of cancer types, linked to the actions of macrophage products on vasculogenesis and cancer cell survival, motility and metastasis. TNF-α is a macrophage product and a product of some cancer cell types that is also associated with adverse prognosis in clinical and experimental cancers, through enhanced tumour cell growth, survival and metastasis. Macrophages are important targets of TNF-α. We observed that TNF-α partly substituted for the macrophage growth factor, M-CSF, in maintaining macrophage survival by protecting cells from apoptosis. We found that TNF-α afforded similar protection to chemotherapeutic agents and related cytotoxic drugs that acted through a range of apoptosis-initiating pathways, but not where protein synthesis was inhibited. Protection was dependent on intact NF-κB signalling. In addition to NF-κB-dependent factors previously identified as anti-apoptotic, we found an absolute requirement for very early antagonism of mitochondrial cytochrome C release, which sufficed to prevent apoptosis in the face of activation of a range of upstream apoptosis pathways, including p53, DISC-linked, mitochondrial depolarisation and calcium-sensitive pathways. The capacity of TNF-α to preserve macrophage numbers in the face of chemotherapy drugs is a potential contributor to prognosis in TNF-α-expressing cancers, encouraging further testing of anti-TNF-α treatments in these patients.

1. Introduction

Macrophages (tumour-associated macrophages: TAM) are present in solid tumours, as part of a chronic tumour inflammatory response. Macrophages theoretically could provide either impetus or hindrance to cancer growth and metastasis. In human cancers, higher TAM numbers are reported to correlate with poorer prognosis in breast, prostate, endometrial, bladder, kidney and oesophageal cancers, and in follicular lymphoma and uveal malignant melanoma, though better prognosis is reported in stomach and colorectal cancers and skin melanoma [1]. In experimental cancer, macrophage presence and activity generally correlates with poorer outcome, through enhanced growth,

Abbreviations: TNF-α, tumour necrosis factor-alpha; NF-κB, nuclear factor-kappa-light-chain-enhancer of activated B cells; DMEM, Dulbecco's modified eagle medium; FCS, foetal calf serum; BMDM, bone marrow-derived macrophages; M-CSF, macrophage colony stimulating factor; TAM, tumour-associated macrophage; DHE, dihydroethidium; $O_2^{\cdot-}$, superoxide anion; Fluo-4-AM, Fluo-4 acetoxylmethyl ester; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAR, parthenolide; 6-MP, 6-mercaptopurine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; RPLPO, ribosomal protein, large, PO; Arg-1, arginase-1; NOS2, nitric oxide synthase 2, inducible; IL-4, interleukin-4; LPS, lipopolysaccharide; IFNγ, interferon gamma; MPT, mitochondrial permeability transition; Cyt C, cytochrome C; CsA, cyclosporin A; Mn-SOD, manganese superoxide dismutase; cIAP-1, cellular inhibitor of apoptosis 1; XIAP, X-linked inhibitor of apoptosis protein; Bcl-2, B-cell lymphoma 2.

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invasiveness, angiogenesis and metastasis, and failure to support adaptive immunity [1]. TAM generally adopt a M2 phenotype, consistent with capacity to enhance cancer growth, promote vasculogenesis and suppress adaptive immunity [2]. Cytokines and chemokines secreted by the tumour cells and hypoxia within poorly-vascularised areas of tumour provide signals for TAM recruitment, phenotype acquisition and survival [1].

TNF-α is produced most abundantly by activated monocyte/macrophage lineage cells, but also by lymphoid, fibroblast, endothelial and mast cells and by some tumour cells [3]. Although it activates apoptosis under some circumstances, cancer cells generally resist TNF-α-induced apoptosis [4]. TNF-α has well-described, but not universal, actions in the promotion and progression of human and experimental tumours [3,5]. TNF-α also acts on TAM promoting a tumour-enhancing phenotype [6] and increasing oxy-radical damage to macromolecules [7]. The canonical NF-κB pathway is of particular importance in transducing TNF-α signals in cancer cells and macrophages [3,8]. TAM exhibit active NF-κB signalling [9]. Transcriptional targets of NF-κB include genes regulating the cell cycle, cell survival, cytokines, chemokines and their receptors. Nuclear expression of the RelA/NF-κB$_1$ (p65/p50) heterodimer confers apoptosis resistance on differentiating macrophages [10]. In other cell types, NF-κB activity has been shown to enhance resistance to chemotherapy-induced apoptosis [11]. These observations led us to propose that TNF-α activity in tumours may make macrophages resistant to apoptosis during chemotherapy, and that this may be through NF-κB activation.

We therefore examined the effects of TNF-α on survival of macrophage cells treated with cancer chemotherapy agents and explored the interactions between TNF-α intracellular signalling and apoptosis pathways. Apoptosis proceeds through two main pathways, termed the extrinsic (death receptor-mediated) and intrinsic (mitochondria-mediated) pathways. Ligation of death receptors, such as TNF-α, Fas or TRAIL receptors, induces assembly of a death inducing signalling complex (DISC) containing FADD (Fas-associated death domain), TRADD (TNFR-associated death domain) and procaspase-8. Auto-activation of caspase-8 occurs, which initiates activation of downstream effector caspases-3/6/7 to ensure the rapid disintegration of the dying cell [12]. In some cell types, caspase-8 mediates the cleavage of Bid, a pro-apoptotic Bcl-2 family member, which translocates to mitochondria to trigger the mitochondria-mediated pathway to apoptosis [12]. Death signals arising from other sources, including signalling by the DNA damage sensor p53, endoplasmic reticulum stress and associated calcium overload, high reactive oxygen production, and reduced ATP levels, also converge on mitochondria to cause apoptosis [13,14]. Intrinsic apoptosis is characterised by the release of mitochondrial proteins, including cytochrome C (Cyt C), second mitochondrion-derived activator of caspase (Smac/DIABLO), apoptosis-inducing factor (AIF) and endonuclease G. Such permeabilisation, indicated by the loss of mitochondrial membrane potential, is thought to be mediated by opening of the mitochondrial permeability transition (MPT) pore that is regulated by pro- and anti-apoptotic members of the Bcl-2 family [15]. In the cytosol, Cyt C assembles with Apaf-1, ATP and procaspase-9 to form the apoptosome. Caspase-9 activation ensues, which then activates effector caspases to complete apoptosis. Endogenous caspase inhibitors include inhibitors of apoptosis (cIAP-1, cIAP-2, XIAP) and FLICE-inhibitory protein (FLIP) [16]. As a terminal caspase in both pathways, caspase-3 activity provides a useful measure of apoptosis, detectable using artificially synthesised DEVD peptide substrates. Annexin-V staining is also a useful early indicator, reflecting the general inversion of phosphatidylserine during apoptosis [17].

2. Materials and methods

2.1. Reagents

Recombinant M-CSF was from R&D Systems. Annexin-V conjugated to phycoerythrin (Annexin-V-PE) and 7-amino-actinomycin (7-AAD) were from BD Biosciences. Parthenolide was purchased from Alexis Biochemicals. Dihydroethidium (DHE) and Fluo-4-AM were purchased from Invitrogen Molecular Probes. All other reagents were sourced from Sigma–Aldrich, unless otherwise indicated.

2.2. Cell culture

RAW$_{264.7}$ cells were obtained from American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (Life Technologies) with 10% low endotoxin fetal calf serum (CSL, Melbourne, Australia), penicillin and gentamicin (DMEM–FCS). Bone marrow-derived monocytes/macrophages (BMDM) were collected from the femora and tibiae of 8-week old female C57BL mice, as previously described [18], and expanded in M-CSF (30 ng/mL)-supplemented DMEM-FCS for 7 days at 37 °C/5% CO_2/95% air. In all cases, cells were cultured for 48 h prior to experimentation.

2.3. MTT cytotoxicity assay

Viability was estimated using the MTT assay, as described by Hu et al. with some minor alterations [19]. BMDM (3×10^4 cells/well) were seeded in 24-well plates in M-CSF-supplemented DMEM–FCS. After 48 h, cells were treated with TNF-α for 3 h, followed by MTX exposure for 24 h. At the end of treatments, 50 μL of MTT (5 mg/mL) was added to each well, and cells were incubated at 37 °C for 2 h. Formazan crystals were then solubilised with 150 μL of 44% dimethyl formamide/20% SDS at room temperature for at least 30 min on a rocking platform. 100 μL aliquots were quantitated at 550 nm using a microplate reader (POLARstar OPTIMA, BMG, Germany). Triplicate assays were conducted for all conditions.

2.4. Caspase-3 protease activity

RAW$_{264.7}$ cells and BMDM were cultured in 6-well plates at a density of 0.3×10^6 cells/well for 48 h. After treatments, trypsin-detached RAW$_{264.7}$ cells and scraped