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Role of leukemia inhibitory factor in nasopharyngeal carcinogenesis

Shu-Chen Liu and Yu-Sun Chang*

Molecular Medicine Research Center; Chang Gung University; Taoyuan, Taiwan

Keywords: leukemia inhibitory factor, p70S6K, mTOR, nasopharyngeal carcinoma, radioresistance, Epstein-Barr virus

Abbreviations: ATM, ataxia telangiectasia mutated; CDC25C, cell division cycle 25C; CTAR, cytoplasmic C-terminal activation regions of LMP1; DDR, DNA damage response; DSB, DNA double-strand break; EBV, Epstein-Barr virus; ERK1/2, p44/42 mitogen activated protein kinase; γH2AX, phosphorylated (Ser139) histone H2AX; Gp130, glycoprotein 130; IL-1, interleukin 1; IL-6, interleukin 6; IL-8, interleukin 8; IL-10, interleukin 10; JAK, Janus tyrosine kinase; LIF, leukemia inhibitory factor; LIFR, leukemia inhibitory factor receptor; LMP1, latent membrane protein 1 of EBV; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; NBS1, nibrin; NPC, nasopharyngeal carcinoma; NRF2, nuclear factor erythroid 2-related factor 2; p70S6K1, 70 kDa ribosomal protein S6 kinase 1; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; sLIFR, soluble leukemia inhibitory factor receptor; STAT3, signal transducer and activator of transcription 3; TGFβ1, transforming growth factor beta-1; TNFα, tumor necrosis factor alpha

Although Epstein-Barr virus-associated nasopharyngeal carcinoma (NPC) is a highly radiosensitive cancer, approximately 20% of patients with NPC develop local recurrence after radiation therapy. Multiple proinflammatory cytokines are thought to protect NPC tumor cells from immune surveillance and therapeutic interventions. The cytokine leukemia inhibitory factor (LIF) is a critical component of the NPC microenvironment. LIF influences tumor growth and survival, and is therefore considered a potential therapeutic target and/or prognostic predictor for NPC. High LIF levels have been detected in the circulating blood of patients with recurrent NPC and NPC tumor cells. This review discusses the molecular mechanisms that link LIF to NPC tumor progression and radioresistance.

Introduction

In nasopharyngeal carcinoma (NPC), genetic alterations and microenvironmental factors such as Epstein-Barr virus (EBV) and infiltrated leukocytes are considered to be major contributors to tumor growth, immune escape, and survival.[1,2] The progression of EBV-associated NPC is profoundly affected by various proinflammatory cytokines that are secreted by tumor cells and surrounding stromal cells, which include lymphocytes, macrophages, T cells, and fibroblasts.[3-6] In chronic inflammatory cancers, proinflammatory cytokines (e.g., interleukins IL-6 and IL-8) play critical roles in tumor progression and resistance to treatment, including radioresistance and chemoresistance.[7-9] NPC is generally sensitive to radiation therapy, and most NPC patients can be cured if the disease is diagnosed and treated at an early stage. However, some NPC patients develop local recurrence after radiotherapy[10] even though they have the same histologic diagnosis as other patients who are cured by the treatment. Although clinical parameters (e.g., a larger tumor volume or advanced tumor stage) may explain some of these treatment failures, many are unexplained. Radioresistance has been recognized as a major cause of treatment failure. Given our current understanding of the determinants of tumor response to radiotherapy, it seems worthwhile to investigate the roles of specific cytokines in tumor radioresistance.

Leukemia inhibitory factor (LIF) is a key factor in the growth of mouse embryonic stem cells and a critical regulator of embryonic development in humans.[11] LIF is a member of the IL-6–type cytokine family, which includes IL-6, IL-11, oncostatin M, ciliary neurotrophic factor, cardiotrophin-1, and cardiotrophin-like cytokine.[12] The receptor for LIF is a heterodimer of the specific gpl90 subunit (LIFR) and the gpl30 subunit. LIF regulates the proliferation, survival, and differentiation of cells by activating critical signaling pathways, including the Janus tyrosine kinase/signal transducer and activator of transcription 3 (JAK/STAT3), p44/42 mitogen activated protein kinase (ERK1/2), and phosphoinositide 3-kinase (PI3K) signaling pathways.[13-15] Although dysregulation of LIF and/or LIFR has been reported in several human malignancies, both prosurvival[16-19] and tumor suppressor[20,21] properties of LIF have been described. Thus, the cancer-related mechanisms of LIF are likely to be complex.

In this review, we address several issues related to the roles of LIF in NPC progression[22] and how LIF is regulated in the NPC tumor microenvironment. We also discuss the potential of LIF as a prognostic biomarker for predicting the radiosensitivity of NPC tumors and as a therapeutic target for this cancer, with the aim of stimulating further translational research on this multifunctional cytokine.

*Correspondence to: Yu-Sun Chang; Email: ysc@mail.cgu.edu.tw
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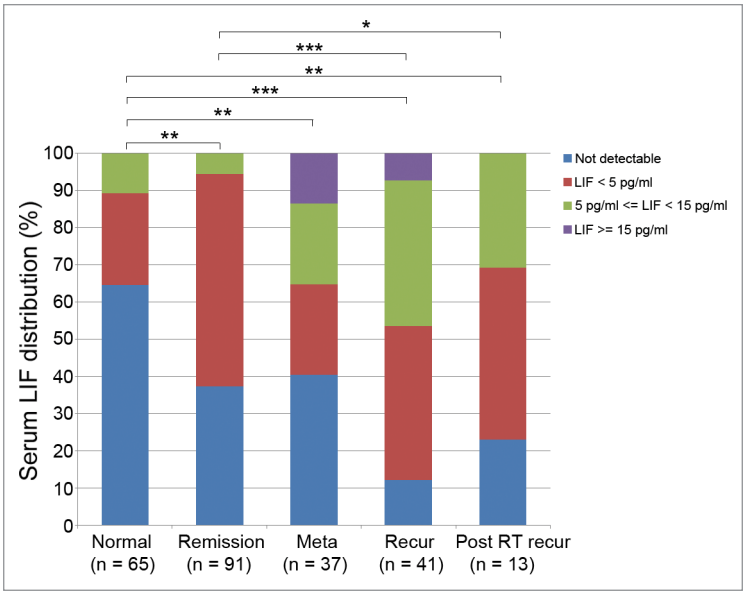

Figure 1. Serum leukemia inhibitory factor (LIF) levels in patients with nasopharyngeal carcinoma (NPC) and normal donors (*$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$; Mann–Whitney test). Meta, metastatic; recur, recurrence; post RT recur, post radiotherapy recurrence.

LIF Levels in NPC

Numerous studies have detected human LIF in the circulatory system or body fluids.[23-29] Elevated LIF levels are associated with inflammation, autoimmune diseases, blastocyst implantation, and cell proliferation.[30-34] LIF can be produced by immune cells and other stromal cells and is induced by various inflammatory factors, including NFκB, TNFα, and IL-1.[35] In patients with type III NPC (also known as lymphoepithelioma), the levels of LIF in the circulating blood and the tumor microenvironment are thought to be associated with tumorigenesis.[22]

LIF in serum samples from NPC patients

The first clue to the clinical involvement of LIF in NPC came from the observation that LIF levels are elevated in serum samples from patients diagnosed with recurrent NPC. In contrast, serum LIF is not detected in approximately 65% of normal individuals (**Fig. 1**). In NPC patients, a increased serum LIF level (LIF > 4.96 pg/mL) is an independent prognostic factor for recurrence-free survival,[22] suggesting that the serum LIF level may serve as a predictor for a patient's response to radiation therapy. However, LIF levels do not differ significantly between patients with metastasis and those with tumor remission (**Fig. 1**), suggesting that the paracrine or juxtacrine regulation of LIF in the tumor microenvironment might be more important than its systemic influence. Furthermore, serum LIF levels in pre-treatment NPC patients are not significantly associated with gender, age, or pathologic classification.

Immunohistochemical analysis of LIF expression

Human LIF exists in at least three isoforms, LIF-D, LIF-M, and LIF-T,[36-38] which differ in their first exons: the *LIF-D* transcript encodes a soluble LIF that is secreted and transduces signaling via LIFR, LIF-T is a truncated isoform found in the nucleus, and the *LIF-M* transcript is translated into both secreted and intracellular proteins. Secreted LIF-M can also be found as a variant that binds to the extracellular matrix.[39] Our immunohistochemical data show that NPC cells express markedly higher levels of LIF compared with adjacent normal epithelial cells. In general, little to no LIF immunoreactivity is found in the nuclei of normal nasopharyngeal basal epithelium cells (**Fig. 2A**), whereas moderate to strong LIF expression is observed in the cytoplasm of nasopharyngeal tumor cells (**Fig. 2B–D**). Strong LIF expression is also correlated with poorer prognosis in our samples. However, further studies are needed to examine whether and how the different LIF isoforms contribute to NPC tumorigenesis. In patients with NPC, macrophages infiltrate into the tumor mass. Our laboratory found that tumor-resident macrophages express very high levels of LIF (**Fig. 2E**), suggesting that macrophages might critically contribute to the high levels of LIF present in the NPC tumor microenvironment.

LIF in the microenvironment of NPC

LIF is secreted by many cell types including macrophages, fibroblasts, mesenchymal stem cells, and cancer cells, and exerts its biologic effects via paracrine and autocrine mechanisms.[40] Type III NPC is characterized by EBV infection and high lymphocyte infiltration,[2] generating a proinflammatory microenvironment that directly influences the fate of tumor cells. LIF levels are markedly higher in NPC biopsies (median value, 135 pg/mg) than in counterpart normal tissue (median value, 41 pg/mg).[22] Moreover, the concentrations of LIF detected in NPC biopsy samples are 10-fold higher than those in serum samples. Thus, LIF produced in the tumor microenvironment likely contributes to the elevated serum levels of LIF detected in NPC patients. Cultured NPC cells secrete LIF in a concentration range of approximately 20 pg/mL to 80 pg/mL.[22] These observations indicate that tumor cells, macrophages, and possibly other stromal cells collectively contribute to the enhanced production of LIF in the NPC tumor microenvironment.

Functional Roles of LIF in NPC

LIF enhances NPC tumor growth

LIF regulates cell proliferation in various types of human cancer.[19,22,41-43] In NPC, LIF increases DNA synthesis and enhances tumor growth. Treatment with soluble LIFR (sLIFR) can counteract the enhancing effect of LIF on cell growth, and treatment with LIF (10 ng/mL) decreases the doubling time of NPC cells by approximately 20%. In a mouse xenograft tumor model, LIF accelerates tumor growth, whereas sLIFR causes growth arrest.[22] Future work is needed to define how LIF alters the matrix structure. However, the LIF-induced enhancement of NPC cell growth might be explained by activation of pro-survival signaling-mediated translational control (see "LIF-mediated signaling in NPC" below).

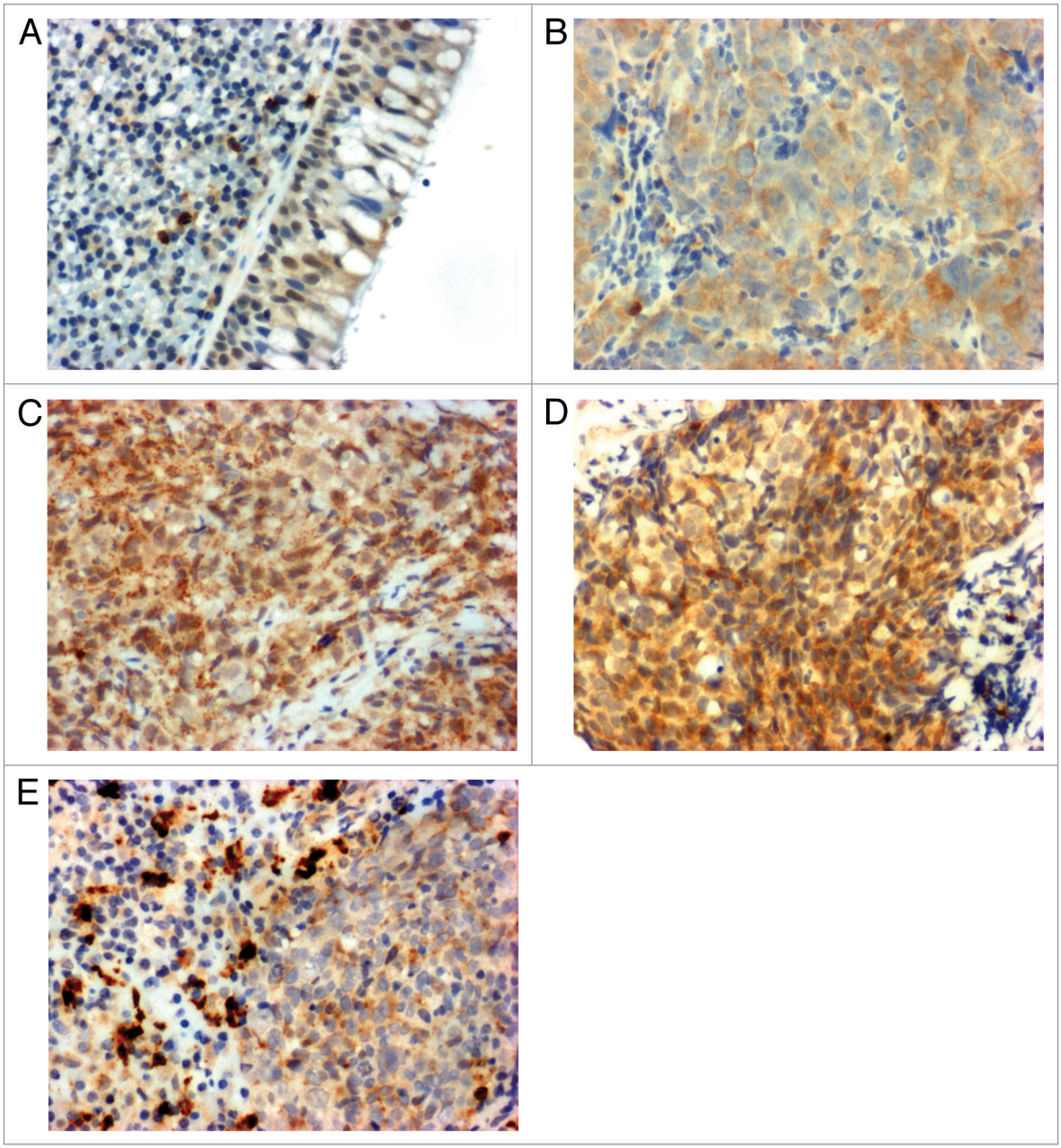

Figure 2. Leukemia inhibitory factor (LIF) expression in nasopharyngeal tissues. (**A**) Normal basal epithelium. (**B**) Nasopharyngeal carcinoma (NPC) tumor diagnosed as complete tumor remission after therapy. (**C**) NPC tumor diagnosed with relapse after therapy. (**D**) NPC tumor diagnosed with distant metastasis after therapy. (**E**) Macrophages residing within NPC tumor areas.

LIF induces dedifferentiation of NPC cells

The role of LIF in differentiation has been well documented in numerous cell types,[41,44-48] but few studies have focused on its involvement in cancer cell differentiation. Recently, we found that LIF can modulate the expression of genes involved in epithelial differentiation. Supplementation of culture medium with LIF alters the morphology of NPC cells to a more undifferentiated phenotype, whereas the addition of sLIFR leads to terminal differentiation (**Fig. 3A**). This may be partly explained by increased expression levels of dedifferentiation markers (KRT14, TGM1, TGM2, SFN) and decreased expression levels of differentiation markers (KRT4, KRT 10, KRT36, IVL) (**Fig. 3B**). Together, available data suggest that the presence of LIF in the tumor microenvironment sustains the characteristics of stem cell-like cancer cells.

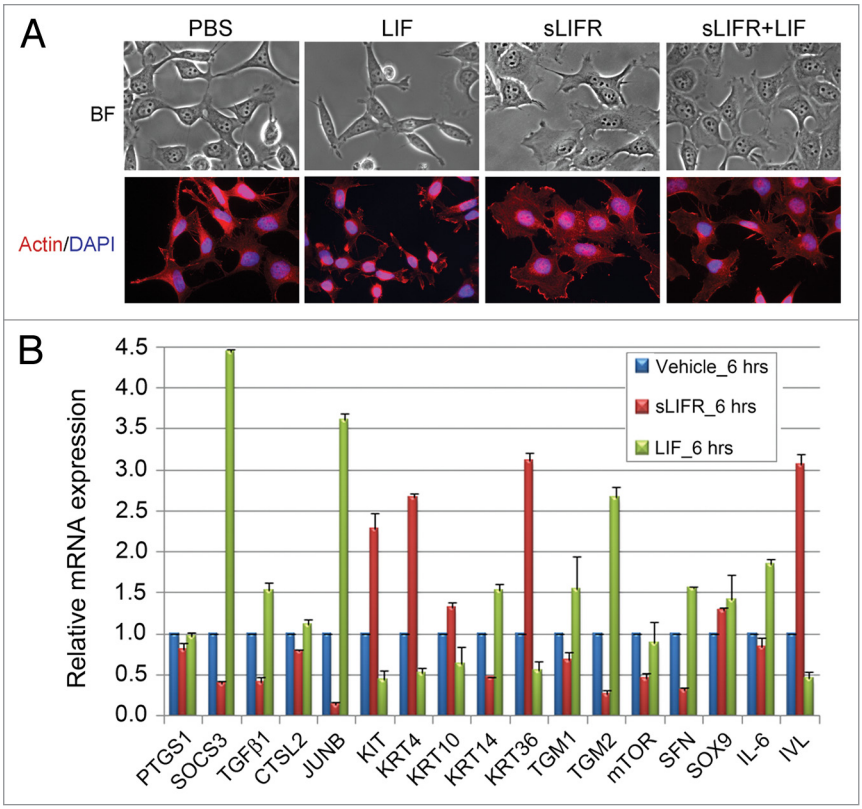

Figure 3. Leukemia inhibitory factor (LIF) treatment induces de-differentiation phenotype of nasopharyngeal carcinoma (NPC) cells. (**A**) Treatment with LIF or soluble LIFR (sLIFR) induces morphologic changes in NPC-TW06 cells. Upper panels, bright field; lower panels, fluorescent staining of actin (red) and DAPI (blue). (**B**) mRNA expression of genes associated with epithelial differentiation by QRT-PCR. Values are presented as means ± SD of duplicate samples.

LIF-Mediated Signaling in NPC

The mTORC1/p70S6K1 pathway is critically induced by LIF in NPC

LIF mediates critical signaling pathways (e.g., the JAK/STAT3, PI3K, and ERK1/2 signaling pathways) to confer cell type- or developmental stage-specific regulation of multiple biologic processes, including cell proliferation, survival, and differentiation.[15,40,49-51] In NPC cells, LIF activates its known targets (e.g., STAT3, MEK1, and p38 MAPK), while additionally activating mTORC1/p70S6K1 signaling. In this pathway, mTORC1 phosphorylates T389 in the linker region of p70S6K1;[52,53] activated p70S6K1 in turn phosphorylates mTOR at Ser 2448 via a feedback loop.[54] Suppression of mTORC1/p70S6K1 with rapalogs or siRNA-mediated silencing of mTOR attenuates LIF-mediated activation of p70S6K1, mTOR, ERK1/2, and STAT3, and decreases LIF-induced NPC tumor growth.[22] In NPC, therefore, mTORC1/p70S6K1 appears to constitute a critical signaling hub that is triggered by LIF. The existence of a LIF-mTORC1-p70S6K1 axis is further supported by close correlations among the levels of LIF, p-p70S6K1 (T389), and mTOR (S2448) found in immunohistochemical analyses of human NPC biopsies and in LIF-treated NPC tumor xenografts.

LIFR contributes to LIF-mediated signaling

Signaling downstream of LIF is mediated by interactions with its two receptor proteins, LIFR and gp130. LIFR belongs to the type I cytokine receptor family. Binding of LIF to LIFR leads to the activation of cytoplasmic tyrosine kinases and triggers intracellular signaling cascades.[50] Receptor activation is followed by desensitization and receptor turnover is under tight control.[55,56] LIFR expression has been reported in a number of human malignancies, including thyroid cancer,[43] rhabdomyosarcoma,[42] pancreatic carcinoma,[19,57] ovarian cancer,[58] breast cancer,[21,59] and hepatocellular carcinoma.[20] In NPC, LIFR expression is significantly higher in the tumor mass compared with the adjacent normal basal epithelium. Ectopic expression of LIFR in cultured NPC cells enhances the activation of downstream signaling molecules in the absence of ligand stimulation, suggesting that overexpression of LIFR is sufficient to trigger signal transduction, possibly mimicking the ligand-LIFR interaction. Consistent with this observation, siRNA-mediated depletion of LIFR suppresses the LIF-mediated activation of downstream signaling (unpublished observations). NPC tumor cells also express high levels of gp130, but these levels are not significantly different from those observed in normal basal cells.[22]

LIF Enhances the Resistance of NPC to Ionizing Radiation

Role of LIF in NPC recurrence

Approximately 20% of NPC patients develop local recurrence after radiotherapy, and the relapsed NPC is usually more advanced than the original tumor.[10] Various proinflammatory cytokines produced by macrophages, epithelial cells, fibroblasts, and cancer cells in the tumor microenvironment (e.g., transforming growth factor β-1 [TGF-β1], tumor necrosis factor α [TNF-α], interleukin 1 [IL-1], IL-6, COX-2, IL-8 and IL-10) critically modulate the efficacy of radiotherapy.[60,61] These proinflammatory cytokines contribute to radioresistance by activating various survival pathways, including the EGFR, STAT3, and PI3K/AKT pathways. LIF levels are elevated in both pretreatment serum samples from NPC patients that developed local recurrence after treatment and in serum samples from post-treatment NPC patients diagnosed with recurrence.[22] Consistent with this finding, administration of LIF enhances the radioresistance of NPC xenografts in immunodeficient mice. The LIF-induced activation of prosurvival signaling (e.g., mTORC1/p70S6K1and STAT3 signaling) in NPC cells may explain its role

in radioresistance. The high level of LIF may serve a radioprotective function against genotoxic insults.

LIF modulates the DNA damage response and radiosensitivity

Cellular radiosensitivity is influenced by both intrinsic factors (e.g., cell cycle distribution, the activation of apoptotic programs, the DNA damage response [DDR], and accumulation of genetic mutations) and extrinsic factors (e.g., oxygen, nutrients, and the elimination of metabolic waste). DNA double-strand breaks (DSBs) are the most critical event in ionizing radiation (IR)-induced cell death. In response to IR, ataxia telangiectasia mutated (ATM) protein functions as a central transducer, triggering a DDR cascade to stimulate apoptosis or DNA repair.[62] Activated ATM phosphorylates various checkpoint proteins (e.g., p53, CDC25C, CHK1, CHK2, and NBS1) during all phases of the cell cycle[63] and also phosphorylates histone H2AX at Ser139 (γH2AX).[64] ATM dysfunction results in abnormal checkpoint responses, genomic instability, cancer predisposition, and high sensitivity to IR, as exhibited in AT cells.[65] The presence of LIF in IR-treated NPC cells decreases activation of DDR signaling molecules, including ATM, NBS1, γH2AX, CDC25C, and p53, leading to increased cell survival.[22] Furthermore, LIF inhibits IR-induced apoptosis, as evidenced by reduced levels of active caspase-3 and caspase-7 in LIF-treated cells.[22] These findings suggest that LIF-mediated radioresistance may result from inhibition of DDR signaling and suppression of apoptosis.

LIF protects cardiac myocytes against oxidative stress under the acute stress condition of ischemia-reperfusion.[50] IR stress is known to generate a high level of intracellular reactive oxygen species (ROS). Notably, we found that the presence of LIF in γ-irradiated NPC cells increases the expression of various antioxidant enzymes, including SOD1, SOD2, CAT, PRDX2, and GPX3, and enhances the nuclear localization of nuclear factor erythroid 2-related factor 2 (NRF2), a key transcription factor involved in the antioxidant response (unpublished observations). This antioxidation effect further elucidates the role of LIF in radioresistance of NPC cells.

Blockade of LIF signaling sensitizes NPC cells to ionizing radiation

Based on the signaling pathways activated by LIF, we hypothesized that blockade of LIF-mediated signaling would sensitize tumor cells to radiotherapy. Our laboratory therefore explored whether administration of sLIFR (which prevents cells from responding to LIF) or rapamycin would increase the therapeutic efficacy of IR Notably, sLIFR appears to enhance IR-mediated cell killing both in vitro and in vivo.[22] sLIFR-mediated enhancement of radiosensitivity is also observed in NPC xenografts stably expressing sLIFR. Furthermore, sLIFR treatment enhances IR-induced DRR and apoptosis in NPC cells, whereas suppression of mTORC1/p70S6K1 signaling by rapamycin or everolimus markedly blocks the effects of LIF on cell growth and radioresistance.[22]

The tolerance of normal tissues is the limiting factor for both radiotherapy and chemotherapy. Radiosensitizers such as gemcitabine and nitroimidazole compounds are highly toxic to normal cells. In contrast, sLIFR is a naturally existing protein. As the toxicities of protein drugs are generally much lower than those of chemical compounds, sLIFR could prove useful for the treatment of cancer patients with high serum LIF levels and/or high LIF or LIFR expression levels in their tumor tissues. This could selectively block the effects of LIF in tumor cells with minimal toxicity to normal cells. Additional studies are needed to establish the physiologic effects of sLIFR in humans, but we believe that it will be worthwhile to test the efficacy of inhibiting

Table 1. Molecular events associated with aberrant activation of leukemia inhibitory factor (LIF) signaling in nasopharyngeal carcinoma (NPC)

Ontologic function	Component	Observed alterations
Prosurvival pathway	p70S6K1	Increased phosphorylation (T389, T421, S424)
	mTOR	Increased phosphorylation (S2448)
	STAT3	Increased phosphorylation (Y705)
	ERK1/2	Increased phosphorylation (T202/Y204)
	GSK-3α/β	Increased phosphorylation (S21/9)
DNA damage responses	ATM	Reduced phosphorylation (S1981)
	H2AX	Reduced phosphorylation (S139)
	NBS1	Reduced phosphorylation (S343)
	CDC25C	Reduced phosphorylation (S216)
Apoptosis	p53	Reduced phosphorylation (S392)
	Caspase 3	Reduced expression
	Caspase 7	Reduced expression
Dedifferentiation	SOCS3	Increased expression
	TGFB1	Increased expression
	JUNB	Increased expression
	KRT14	Increased expression
	TGM1	Increased expression
	TGM2	Increased expression
	SFN	Increased expression
	IL-6	Increased expression
	KRT10	Reduced expression
	KIT	Reduced expression
	KRT4	Reduced expression
	KRT36	Reduced expression
	IVL	Reduced expression

LIF-LIFR interactions in a Phase I trial in patients with radiation-treated NPC.

In summary, we have described a number of LIF-induced alterations in NPC. **Table 1** summarizes the molecular events associated with aberrant activation of LIF-mediated signaling in NPC.

Role of EBV in Regulating LIF Production

In EBV-associated NPC, EBV establishes the latency II program, which is characterized by limited expression of viral proteins and RNA.[66] Latent membrane protein 1 (LMP1), the most important EBV-encoded oncoprotein in NPC, functions as a constitutively active tumor necrosis factor receptor (TNFR) by mimicking CD40 signaling.[5] The transmembrane regions and the cytoplasmic C-terminal activation regions (CTARs; CTAR1, CTAR2, and CTAR3) of LMP1 can trigger cell type- and latency stage-specific signaling. The LMP1-mediated signaling pathways are described in detail in several excellent reviews.[67-69] In NPC, the activation of NF-κB is the most critical event in LMP1-mediated signaling. CTAR1 activates NK-κB through TRAF1, TRAF2, and TRAF3, whereas CTAR2 activates NF-κB through TRADD and TRAF2.[70,71] In addition to functioning as a growth promoter for NPC cells, LMP1 also induces multiple immunomodulatory effects that allow tumor cells to circumvent immunosurveillance, at least in part by modulating cytokine expression in the tumor microenvironment.[4,69] Promoter analysis suggests that there are three NF-κB/c-Rel binding sites in the promoter region of LIF, and we found that the CTAR1 and CTAR2 domains of LMP1 both contribute to the production of LIF via activation of NF-κB, whereas C-terminally deleted LMP1 does not confer this effect.[22] These observations suggest that EBV is a key factor in directing the cytokine profile of the NPC tumor microenvironment.

Concluding Remarks

Although overexpression of LIF has been reported in a number of human malignancies, the functions of LIF in human cancers and the underlying mechanisms remain largely unexplored. As we gain a greater understanding of the roles of LIF in NPC we should be able to further elucidate the multifaceted regulation of LIF in cancer. The next step will be to investigate the potential benefits of sequestering LIF in the tumor microenvironment of NPC patients with high LIF levels in peripheral blood or biopsy samples, for example using a LIF antagonist such as sLIFR or a specific monoclonal antibody.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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