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HETEROGENEITY AMONG HUMAN NASOPHARYNGEAL CARCINOMA CELL LINES FOR INFLAMMATORY CYTOKINES mRNA EXPRESSION LEVELS

Yann Mahé[1]*, Kunitaka Hirose[2], Bernard Clausse[1], Salem Chouaib[1], Thomas Tursz[1], and Bernard Mariamé[1]

[1] Laboratoire de Biologie des Tumeurs Humaines, URA 1156 CNRS, Institut Gustave Roussy, 94805 Villejuif, FRANCE

[2] Kureha Chem. Ind. Co, LTD, Shinjuku-ku, Tokyo 169, JAPAN

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Using polymerase chain reaction (PCR), we confirmed the expression of interleukin-1α (IL-1α) by the human nasopharyngeal carcinoma (NPC) cell line C15 without contribution of either human IL-1ß or mouse IL-1α in the biological activity previously found in C15. However we showed that IL-1α was not expressed in all NPCs. IL-1ß and/or tumor necrosis factor (TNF)-α genes could also be activated, independently from the number of Epstein Barr Virus (EBV) copies harbored by the cells. Interestingly, the primary tumor C15 showed a profile of TNF-sensitive tumor while C17, C18 and C19 which were derived from metastasis have a typical profile of TNF-resistant cells. Furthermore, the inflammatory cytokines whose genes are classically induced by IL-1 and TNF were found expressed only in C17 and C19 suggesting another level of heterogeneity among NPCs.

NPC is a human epithelial tumor with a high rate of incidence in Southeast Asia, and to a lesser extent in North Africa, East Africa and in Artic regions. These malignant cells always harbor the EBV genome (1) and the tumors are heavily infiltrated with non-malignant tumor infiltrating lymphocytes (TILs) with cytotoxic potential (2). In a previous report, using the first NPC tumor (C15) that was established on nude mice in our laboratory, Busson et al. described a preferential production of IL-1α in C15 as evidenced by mRNA production and biological activities (3). In order to establish whether this observation could be considered as a general feature of all NPCs, or correlated with the

* To whom correspondence and reprint requests should be addressed.

Abbreviations used in this paper: NPC: NasoPharyngeal Carcinoma; EBV: Epstein Barr Virus; TIL: Tumor Infiltrating Lymphocyte; IL: Interleukin; mRNA: Messenger Ribonucleic Acid; TNF: Tumor Necrosis Factor; MCAF: Monocyte Chemotactic and Activating Factor; PCR: Polymerase Chain Reaction; MnSOD: Manganous Superoxyde Dismutase.

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expression of a precise EBV gene product in NPCs, we started experiments, using PCR, on three other NPC cell lines (C17, C18 and C19) which were also established in our laboratory to define the pro-inflammatory (i.e.,IL-1α, IL-1ß and TNFα) and inflammatory (i.e., Monocyte Chemotactic and Activating Factor (MCAF), IL-6 and IL-8) cytokines expression profiles of those four NPCs.

MATERIALS AND METHODS

Nude mouse transplantable NPC tumors: Four NPC tumors have been established in our laboratory (3, 4,). Briefly, C15 , C17, C18 and C19 are permanently transplantable NPC tumors from North African patients. In all tumors, established for over 7 years (C15), 4 years (C17, C18) and 3 years (C19) the neoplastic tissue which has been transplanted subcutaneously in swiss nude mice lost infiltrating lymphocytes of human origin, while only malignant cells survived and proliferated in the transplanted mice.

mRNA preparation: Total cellular RNA was extracted according to the method of Chirgwin (5) using cesium-trifluoroacetate (Pharmacia, Upsala, Sweden) instead of Cesium Chloride. 1 μg of RNA (poly-A+ for C15, C17, C18 and total RNA for C19) was then reverse-transcribed using super script reverse transcriptase and oligo(dT) primers according to the manufacturer's instructions (Gibco-BRL, Bethesda, MD).

PCR reaction and primers: 1/20 th. of the cDNA obtained was used for PCR using the indicated primers at a final concentration of 1μM. IL-1α primers were purchased from Clontech Laboratories Inc. (Palo Alto, CA). All primers and expected size of the product of amplification are described in Table 1. PCR was achieved in a final volume of 50 μl (1 cycle at 94°c, 5 min. followed by 30 cycles: 94°c, 1 min.; 55°c, 1 min.45 s.; 72°c, 1 min.30 s. followed by 1 cycle at 72°c for 5 min.) with 2.5 units of Taq DNA polymerase in the buffer conditions recommended by the manufacturer (Promega, Madison, WI) and in the

TABLE 1
LIST OF PRIMERS USED FOR PCR

		Expected size of PCR product	Intron spanning primers
hIL-1α	5'primer: 5'-ATGGCCAAAGTTAGATGTTTG-3'		
hIL-1α	3'primer: 5'-GGTTTTCCAGTATCTGAAAGTCAGT-3'	816 bp	Yes
mIL-1α	5'primer: 5'-ATGGCCAAAGTTCCTGACTTGTTT-3'		
mIL-1α	3'primer: 5'-CCTTCAGCAACACGGGCTGGTC-3'	625 bp	?
hIL-1ß	5'primer: 5'-AGCTGATGGCCCTAAACAGATGA-3'		
hIL-1ß	3'primer: 5'-GATCTACACTCTCCAGCTGTAGA-3'	533 bp	Yes
hTNF-α	5'primer: 5'-CTTCTGCCTGCTGCACTTTGGA-3'		
hTNF-α	3'primer: 5'-TCCCAAAGTAGACCTGCCCAGA-3'	547 bp	Yes
hIL-6	5'primer: 5'-TATCTCCCCCTCCAGGAGCCCA-3'		
hIL-6	3'primer: 5'-AACAACAATCTGAGGTGCCCATGC-3'	706 bp	Yes
hIL-8	5'primer: 5'-GCTTCTAGGACAAGAGCCAGGAAG-3'		
hIL-8	3'primer: 5'-CTTGGATACCACAGAGAATGAATTTTT-3'	406 bp	Yes
hMCAF	5'primer: 5'-GAAACATCCAATTCTCAAACTGAAGC-3'		
hMCAF	3'primer: 5'-TTTGGGTTTGCTTGTCCAGGTGGT-3'	331 bp	?
hTNF-R	5'primer: 5'-CCCACTCCAGGCTTCACCCCC-3'		
hTNF-R	3'primer: 5'-AAAAGTGGGGTTGGAAGGCGATCT-3'	608 bp	?
hMnSOD	5'primer: 5'-CAGCATGTTGAGCCGGGCAG-3'		
hMnSOD	3'primer: 5'-TTATACTGAAGGTAGTAAGCGTGC-3'	585 bp	?
hß-Actin	5'primer: 5'-ATGGATGATGATATCGCCGCGCGCT-3'		
hß-Actin	3'primer: 5'-CGGACTCGTCATACTCCTGCTTG-3'	1067 bp	Yes

presence of 2.5 mM MgCl2. 20 μl of the PCR products were electrophoresed on a 1% agarose gel in Tris Buffer-EDTA buffer 1X, 1 hour and visualized under ultra-violet light after ethidium bromide staining.

RESULTS AND DISCUSSION

Pro-inflammatory cytokines mRNA expression by human NPCs: As shown in Fig.1, using PCR with the respective cytokines primers, we were able to identify and compare the profiles of pro-inflammatory cytokines mRNA expression of the four different human NPCs cell lines that we have established on nude mice. In human monocytes, there is usually a preferential expression of mRNA for IL-1ß, IL-1α being the minor form of IL-1 produced (6 for a review). However, as previously described using either Northern blotting or blocking anti-IL-1 antibodies against each IL-1 subspecies (3), we do confirm here, using PCR, that C15 preferentially produces IL-1α mRNA. Furthermore, in this tumor, we also report here for the first time that no mRNA coding for the two other major pro-inflammatory cytokines (IL-1ß and TNFα) could be detected.

The three other NPCs showed distinct patterns of cytokines expression: The human NPC cell line C17 clearly showed a production of mRNA for both forms of IL-1 and also for TNFα. In contrast, the expression of those cytokines was completely shut down in C18. In C19, TNFα mRNA could be detected at a high level in the absence of any detectable mRNA coding for the two others pro-inflammatory cytokines. In the same experiment, control PCR using human ß-actin primers showed that comparable amounts of mRNA were used in the different experiments (Fig.1).

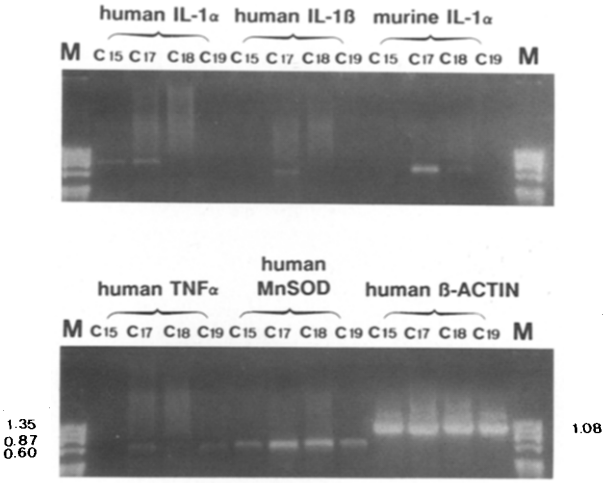

Fig. 1: Pro-inflammatory cytokines mRNA expression in NPC: cDNA from C15, C17, C18 and C19 have been used as template in PCR reaction with the respective primers for either the indicated cytokines or the radical scavenger enzyme, MnSOD, as described in materials and methods; M: size marker, Hae III digest of Phix174.

In the report showing a preferential production of IL-1α in C15 NPC cell line (3), one could not totally exclude the contribution of the major IL-1 subspecies produced by murine monocytes (i.e.,IL-1α,see 6 for a review) in the overall IL-1 biological activity of C15 which was at that time evaluated in the co-stimulatory mouse thymocyte proliferation assay or by IL-2 production by an IL-1 dependent cell line (3). Using primers that specifically allow detection of murine IL-1α message, as shown in Fig.1, we could rule out the contribution of murine IL-1α in C15 and C19. However, RNA isolated from the two other NPC tumors (C17 and C18) clearly contained murine IL-1α message. Furthermore, since there is no correlation between the expression of murine and human IL-1α among the different tumors, an eventual cross-priming of the murine IL-1α primers with human IL-1α reverse-transcripted cDNA is excluded. Whether this IL-1α comes from the endothelial cells of murine origin that contribute to the vascularization of the tumor or comes from murine blood borne cells (i.e., Monocytes/macrophages) remains to be established.

mRNA expression levels for the inflammatory cytokines IL-8 and MCAF: The genes for the inflammatory cytokines IL-8, IL-6 and MCAF are classically inducible by IL-1 and TNF (7, 8, 9 for a recent review). Thus, it was of interest to look for the production of IL-8, IL-6 and MCAF mRNA in NPCs since the expression of these cytokines could reflect an "in vivo" production of biologically active pro-inflammatory cytokines (IL-1 and TNF) in NPCs. As shown in Fig.2, none of these cytokines were found expressed in C15. In contrast, MCAF mRNA could be detected easily in C17 and to a lesser extent in C19 . The latter was also found to express IL-8 mRNA. On the other hand, none of these tumors were found to express IL-6 mRNA.

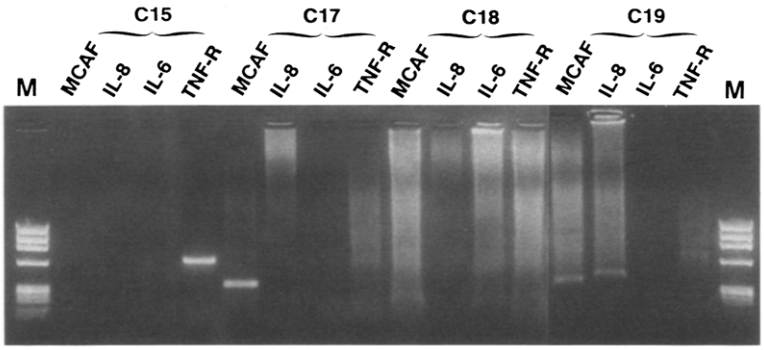

Fig. 2: Inflammatory cytokines and TNF receptor mRNA expression in NPC: cDNA from C15, C17, C18 and C19 have been used as template in PCR with the respective primers as indicated in materials and methods and Table 1; M: size Marker, Hae III digest of phix174.

TNF-receptor (p55) expression in NPCs: TNFα is a pleiotropic cytokine showing several similarities with IL-1 in terms of pro-inflammatory effects and genes activation. However, one of the major specific effect of TNF is its potent ability to induce lysis of tumoral cells either by direct lysis through mechanisms very similar to apoptosis or by a necrosis of neo-tumoral vessels leading to tumor necrosis by a lack of vascularization (9). Additionally, TNF lytic effect is thought to be mediated through the type I (p55) TNF-receptor (10). Surprisingly, in three, out of the four NPCs, we could detect a significant production of TNFα mRNA. On the other hand, among the four NPC tested, TNF receptor (p55) was found expressed only in C15 (Fig.2). This is consistent with the fact that C15 was the only NPC which did not produce mRNA for TNFα (Fig.1) suggesting that this cell line might be a good target for TNF lysis. It is noteworthy that C15 only originates from a primary tumor while the three other cell lines originate from metastases. This observation suggests that an escape of metastatic material from the lytic control of TNFα might occur during the spreading of the disease "in vivo". Additionally, since transfection of TNF-sensitive tumoral cell lines with an expression vector for manganous superoxide dismutase has been reported to protect against the lytic effect of TNF (11), we investigated whether the TNF mRNA producing NPC cells could produce mRNA for MnSOD or not. As shown in Fig. 1 this is the case for C17, and C18 which produce levels of MnSOD mRNA significantly higher than those found in C19 and C15, suggesting that endogenous MnSOD might play a protective role against TNF effects in C17 and C18.

Lack of correlation between mRNA expression for cytokines and EBV genes expression: We tried to establish a possible correlation between cytokine mRNA expression pattern and the mRNA expression of EBV genes. A strong expression of Latent Membrane Protein mRNA could be observed in C15 (and to a lesser extent in C18), while in both C17 and C19 no mRNA for the LMP could be detected (B.M, Y.M., data not shown). This preferential expression of Latent Membrane Protein corellates with the amount of EBV copies detected in the different tumors (n=200 in C15 and C18, n=30 in C19, n=5 in C17, B.M., unpublished results). However, it did not correlate with the expression rate of the cytokines studied here (Fig.1 and 2), suggesting a clear dissociation between the mechanisms controlling the expression of the cytokines in one hand and those involved in viral proteins expression in the other hand. However, the EBV genome contains more than 100 putative genes, and the products of some of them are likely to participate in the immortalization of those EBV-harboring malignant epithelial cells. In conclusion, we suggest that IL-1α over expression previously reported in C15 (3) is not a common feature of all NPCs. However, NPC cells appear to express mRNA for several other cytokines. The role of these cytokines in the interactions between malignant epithelial cells and the important lymphocytic infiltrate regularly found in NPCs remains to be elucidated.

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