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IRF-1 and p65 mediate upregulation of constitutive HLA-A antigen expression by hepatocellular carcinoma cells

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Abstract

Malignant transformation of hepatocytes is frequently associated with upregulation of HLA-A expression. Currently there is no information available regarding the mechanisms underlying this phenotypic change. We investigated HLA-A expression in 165 paraffin embedded tissues and 21 fresh tissues from liver cancer patients. Utilizing truncated HLA-A promoter-reporter constructs and gel-shift assay we had identified the regulatory elements and transcription factors required for HLA-A upregulation. 54% of the paraffin embedded tissues showed increased HLA-A expression in their cancerous part. 43% of the fresh liver cancer tissues had increased HLA-A complex expression with the HLA-A heavy chain gene demonstrating the highest level of upregulation (62%). Enhanced HLA-A expression in the liver cell lines QGY7701 and BEL7402 was found to be mediated by binding of interferon regulatory factor 1 (IRF-1) to interferon stimulated response element, and of nuclear transcription factor p65 binding to enhancer A element in the HLA-A promoter of these cell lines. The in vivo relevance of these findings was indicated by the association of the enhanced expression of IRF-1 and accumulation of nuclear p65 with HLA-A upregulation in 8 of the 21 liver cancer lesions investigated. Our results indicated that HLA-A upregulation in liver cancer was mediated by both increased nuclear aggregation of transcription factor p65 and upregulation of transcription factor IRF-1.

Keywords

Hepatocellular carcinoma; HLA-A antigens; Transcription activation; Interferon regulatory factor-1; Transcription factor p65

1. Introduction

Human Leukocyte Antigen (HLA) class I molecules function as recognition structures in immune response by presenting foreign antigenic peptides to cytotoxic T lymphocytes (CTL). Downregulation or loss of HLA class I antigen has been found in 16–50% of

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2009.03.001.

malignant lesions and is believed to be a strategy by which cancer cells escape immune surveillance (Chang et al., 2003). In some tumor types, HLA class I downregulation is associated with histopathological markers of poor prognosis and reduces tumor-free survival (Ogino et al., 2006; Rolland et al., 2007). Various mechanisms have been proposed and tested for the downregulation or loss of HLA class I in tumor cells. Total loss of HLA class I expression has been reported in some tumors because of defects of $\beta 2$ microglobulin ($\beta 2m$) or HLA class I α chain gene (Raffaghello et al., 2005; Campoli and Ferrone, 2008). Abnormal HLA class I expression can also be due to alterations in the peptide processing machinery, in which numerous intracellular molecules can influence the antigen processing and consequent surface expression of HLA class I molecules in malignant cells (Mehta et al., 2008). Interestingly, hepatocellular carcinomas (HCC) and leukemia (Wetzler et al., 2001) are the two exceptions to these general findings of HLA class I downregulation or loss in malignant lesions. As we previously reported (Bi et al., 2002), normal hepatocytes not expressing or expressing very low levels of HLA class I antigens acquired the expression of these antigens during malignant transformation. To date, defects in HLA class I antigen expression in malignant liver cells have only occasionally been identified.

Like their counterparts in other animal species, HLA class I antigens are constitutively expressed on most, but not all nucleated cells (Bodmer et al., 1993). HLA class I antigen is a cell surface glycoprotein composed of a heavy chain, $\beta 2$ microglobulin and a peptide. Any defect in these components or proteins involved in antigen processing progress, such as the transporter proteins associated with antigen-processing, TAP-1 and -2 (Pandha et al., 2007), and a TAP-associated protein, Tapasin (Facoetti et al., 2005), can result in the downregulation or loss of HLA class I antigen. Among these components, the regulatory elements and the corresponding transcription factors that maintain constitutive HLA-A heavy chain expression have been identified (Blanchet et al., 1994). Three major regulatory promoter elements mediate different routes of tissue specific and cytokine induced transcription of HLA-A gene: the enhancer A; interferon stimulated response element (ISRE); the site α and enhancer B (also named SXY module). These have been mapped to a region extending from -220 to -95 upstream of the transcription initiation site (Griffioen et al., 1999). These regulatory promoter elements with their corresponding transcription factors have been reported to be involved in HLA-A downregulation in different types of tumor cells. For example, it has been showed that downregulation of HLA-A gene transcription is controlled by the proximal promoter element in choriocarcinoma cells (Lefebvre et al., 1999). However, the role of these regulatory promoter elements and their corresponding transcription factors in the upregulation of HLA-A expression described in liver tumor cells remains to be determined.

In this study, we aimed to identify regulatory sequences and transcription factors involved in HLA-A antigen upregulation in hepatocellular carcinomas. We were able to demonstrate that Hepatocellular Carcinoma (HCC) exhibited upregulation of HLA-A antigens. Enhanced expression of the HLA-A heavy chain was mainly mediated by increased nuclear translocation of transcription factor p65 and upregulated expression of transcription factor IRF-1 in HCC cell lines and tissues. To our best knowledge, this is the first report of the molecular mechanisms associated with constitutive HLA class I upregulation in human hepatocellular carcinomas. Since HLA class I antigens are important in the recognition of tumor cells by tumor-specific CTL, down-regulation of HLA class I antigens in malignant cells provides a way by which tumors can escape T-cell surveillance, adversely affecting the result of clinical T-cell-based immunotherapy (Masuda et al., 2007). Gaining more knowledge of HLA class I antigens expression status in HCC and the molecular mechanisms associated with it will be an important factor in selecting patients suited for the adoptive T cell therapy in clinical trials.

2. Materials and methods

2.1. HCC specimens

After obtaining informed consent at Qidong Liver Cancer Institute (Jiangsu province, China) and Jiangsu Provincial People's Hospital (Jiangsu province, China), 165 formalin-fixed paraffin-embedded liver tissue specimens were collected from patients with a histologically confirmed diagnosis of primary HCC between 1989 and 2003 (144 male, 21 female, age range 30–80 years), and 21 fresh liver tissue samples were obtained from liver cancer patients who had undergone surgical resection without any prior radio- or chemotherapy from 2004 to 2005 (18 male, 3 female, and age range 33–66 years). Surgically removed biopsies were immediately snap-frozen in liquid nitrogen and stored at -70°C for up to 6 months. All these samples were reviewed by a pathologist to confirm the diagnosis of HCC.

2.2. HCC cell lines

HCC cell lines BEL7402, BEL7405, and QGY7701 were obtained from the cell bank of the Type Culture Collection of the Chinese Academy of Science (Shanghai, China). Cells were cultured in RPMI1640 medium (Gibco-BRL, Grand Island, NY, USA) and supplemented with 10% fetal bovine serum (Gibco). Recombinant IFN- γ was purchased from Pep Rotech EC Ltd, London, UK.

2.3. Antibodies

Mouse monoclonal antibodies (mAb) HCA2 (Sernee et al., 1998), LGIII-147.4.1 (Wang et al., 2003), and the isotype-matched irrelevant mAb MK2-23 (Chen et al., 1993) were characterized as previously described. Anti-IRF-1, anti-p65, anti-I κ B α , anti-c-myc, anti- β actin mAb, and FITC-conjugated secondary antibodies were purchased from BD Biosciences (San Jose, CA, USA). Anti-IRF-1, anti-IRF-2, and anti-p65 mAb used for gel-shift assays were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

2.4. Immunohistochemistry

Snap-frozen HCC biopsies were embedded and cut into 6 μm sections, and paraffin-embedded HCC tissues were serially cut into 4 μm sections. Immunohistochemical staining was performed with the avidin–biotin–horseradish peroxidase complex (ABC) detection method (Vector Laboratories, Burlingame, CA, USA). All immunostained sections were blindly examined with a light microscope by two investigators. Kuffer cells, endothelial cells, and tumor-infiltrating lymphocytes on each slide were used as positive controls. Staining with isotype-matched irrelevant mAb was used as negative controls. Immunostained cases were scored as 0 (negative or occasional positive cells), 1+ (<10% positive cells), 2+ (10–25% positive cells), 3+ (26–50% positive cells), 4+ (51–75% positive cells) or 5+ (>75% positive cells). Based on this scoring system, the expression of HLA molecules in cancer cells was compared with the staining of their normal corresponding parts and described in three ways: upregulation, consensus expression and downregulation.

2.5. Flow-cytometry analysis

Cells were washed twice with phosphate-buffered saline (PBS) 48 h after transfection, detached from culture dishes with 0.25% trypsin, washed, and resuspended in PBS supplemented with 1% bovine serum albumin (BSA). Approximately 5×10^5 cells were incubated with mAb LGIII-147.4.1 and then with FITC labeled secondary antibodies for 30 min at 4°C . Control aliquots were stained with an isotype-matched irrelevant antibody and with the same secondary antibody. Analysis was performed using a fluorescence-activated cell sorter (FACS; calibur, BD, USA). Fluorescence intensity was evaluated by histogram

analysis and was expressed as Mean Fluorescence Intensity (MFI) which represented the average fluorescence intensity of the main cell population investigated.

2.6. Semi-quantitative reverse transcription polymerase chain reaction assays

Total RNA was extracted with Trizol (Invitrogen, San Diego, CA, USA) and reverse transcribed using RT-PCR kits (Promega, Madison, WI, USA) according to the manufacturer's protocol. PCR products were amplified in a 25 μ l mixture. The amplification programs and primers used for amplification of HLA-A, β_2 m, TAP1, TAP2, Tapasin, and β -actin (Xia et al., 2007) were previously confirmed for the detection of each gene. The sizes of the PCR products were verified to the predicted sizes by 2% agarose gel electrophoresis and each of the PCR analyses was repeated at least three times.

2.7. Western blot assays

The whole cell lysates were obtained by RIPA buffer treatment with protease inhibitors. Nuclear protein was prepared by NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA). Protein concentration was quantified by the Bradford assay (Pierce) using BSA as standard. After centrifugation, 20 μ g of total protein were denatured at 100 °C for 5 min in a protein sample buffer, separated on 10% polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE), and transferred to a nitrocellulose membrane (Roche) for immunoblotting. Primary antibodies used were anti-HLA-A heavy chain (HCA2), anti-IRF-1, anti-p65, anti-I κ B α , anti- β actin, and anti-c-myc. Immunoblot analysis was performed using HRP-conjugated goat anti-mouse antibodies and developed using the enhanced chemiluminescence (ECL) kits (Amersham Biosciences, Little Chalfont, UK). Immunospecific bands were quantified by using an optical densitometer and normalized with the intensity of the β -actin band.

2.8. Reporter and expression plasmids

The pGL3-A217, pGL3-A200, pGL3-A184, pGL3-A161, and pGL3-A96 reporter plasmids were generated by PCR and subsequently cloned as a 217-bp, 200-bp, 184-bp, 161-bp, and 96-bp HLA-A*0201 promoter fragment upstream of the firefly luciferase gene in pGL3-Basic Vector (Promega). For the deletion of ISRE construct, the deleted base pairs were CGCAGTTTCTTTCTCC, which span -185 to -169 of the HLA-A promoter. pcDNA3.1-IRF-1, pcDNA3.1-IRF-2, and pcDNA3.1-p65 were created by PCR amplification of full-length cDNA of IRF-1, IRF-2 and p65 genes from normal lymphocyte cells and ligated into pcDNA3.1-myc-his-A (Invitrogen). A dominant-negative form of IRF-1 was also constructed into pcDNA3.1-myc-his-A as described in a previous report (Bouker et al., 2005). I κ B wild type vectors and I κ B mutant (S32 and S36) were obtained from Dr. Bernd Baumann (Physiological Chemistry, Ulm University, Germany). Substitution of the two serines 32 and 36 in wild type I κ B by alanine residues protects this I κ B mutant from ubiquitination and proteasome-mediated proteolysis. All the constructs were verified by DNA sequencing.

2.9. Transient transfections and luciferase reporter assay

One day before transfection, 2×10^5 HCC cells were seeded in each well of a six-well plate. Using the FuGENE 6 (Roche), the cells were co-transfected with the series of plasmids accompanied by pRL-SV40 (Promega), which served as a control to determine transfection efficiency. After 48 h of transfection, the cells were lysed and luciferase activity was assayed with the Promega Dual Glo assay kit by using a TD 20/20n luminometer (Turner Biosystems, Sunnyvale, CA, USA). The data represented the average of three independent experiments and were shown with the standard error.

2.10. Electrophoretic mobility shift assay (EMSA)

Total nuclear proteins (10 µg) were incubated with 2 ng of a ³²P-labeled dsDNA probe for 30 min in EMSA binding buffer (Promega). The samples were run on a 6% nondenaturing polyacrylamide gel in 0.25× TBE at 200 V for 2 h at 4 °C. Gels were fixed and exposed to an X-ray film (Kodak). For the competition assays, nuclear extracts were first incubated with 100, 200, or 400 ng of unlabeled oligonucleotide for 30 min, and then ³²P-labeled probes were added. Supershift assays were performed by incubating nuclear extracts with 1 µg of antibody for an additional 30 min after incubation with labeled oligonucleotides. The following double strand oligonucleotides were used as probes (Invitrogen):

OCT sequence: 5'-TGTCGAATGCAAATCACTAGAA-3'

kB sequence: 5'-TTGGGGAGTCCCAGCCTTGGGGATTCCCCAACT-3'

ISRE sequence: 5'-CTCCGCAGTTTCTTTTCTCC-3'

2.11. Statistical analysis

Cochran–Mantel–Haenszel statistics were adopted to examine the relationship between the variables. A *p* value <0.05 was considered statistically significant.

3. Results

3.1. Upregulation of human leukocyte antigen class I antigens in hepatocellular carcinoma lesions

The 165 surgically removed, paraffin embedded primary tumor carcinomas and adjacent normal tissues were staged as follows: 1 in stage I, 85 in stage II, and 79 in stage III. When compared to their normal counterparts, 54% of the lesions showed increased HLA-A antigen expression in their cancerous parts, 38% demonstrated consensus HLA-A expression to their normal parts, and only 8% had lower HLA-A expression (Fig. 1A). We did not find any relationship between HLA-A upregulation and pathological grade of the tumor. Increased HLA-A expression was not correlated with tumor size, AFP level, HBV and/or HCV infection, or cirrhosis.

Due to a shortage of antibodies suitable for staining of antigen processing molecules in paraffin embedded tissues, another 21 pairs of fresh liver cancer lesions and their corresponding normal parts were used to detect the expression of the surface HLA-A complex and of several antigen processing components at the same time. Similar to the results in paraffin embedded tissues, 9 of the 21 patients (43%) showed increased HLA-A complex expression in the cancerous parts (Fig. 1B). Furthermore, semi-quantitative RT-PCR showed that the HLA-A heavy chain gene had the highest level of upregulation (62%) in liver cancer. Enhanced expression of β2microglobulin was also detected in 9 patients (43%) and the expression of TAP1, TAP2 and tapasin increased in 10 (50%), 12 (57%) and 8 (38%) patients, respectively (Fig. 1C, Table 1).

3.2. The ISRE and enhancer A were important elements of the constitutive HLA-A promoter activity

To determine the regulatory elements involved in HLA-A heavy chain transcription, a series of truncated HLA-A promoter–reporter constructs were transfected into QGY7701, BEL7402 and BEL7405 HCC cell lines (Fig. 2A), which express a high, medium and low amount of HLA-A heavy chain, respectively (Fig. 2B). As shown in Fig. 2C, the activity of the entire 217 bp HLA-A promoter was elevated in QGY7701 and BEL7402 compared to BEL7405. Deletion of enhancer A resulted in a greater reduction of HLA-A promoter activity in QGY7701 and BEL7402 than in BEL7405 (2.3 and 2.6 folds vs. 1.7 folds).

Similarly, deletion of ISRE resulted in a bigger decrease in HLA-A gene transcription in QGY7701 and BEL7402 compared to BEL7405 (2.5 and 2.3 folds vs. 1.5 folds). Truncation of both enhancer A and ISRE resulted in a marked decrease in promoter activity, with a 21-, 9.7-, and 3.1-fold decrease in QGY7701, BEL7402, and BEL7405, respectively (Fig. 2D). Differences in the promoter activity of the three cell lines were almost absent with deletion of enhancer A and ISRE. These results indicated that the activities of enhancer A and ISRE were obviously increased in QGY7701 and BEL7402 cells compared to BEL7405, with the combined transcriptional activity of both elements being more than that of each individual element.

3.3. IRF-1 regulated HLA-A promoter activity through ISRE

In order to characterize the DNA binding factors that interact with ISRE in the cell lines mentioned above, gel-shift assays were performed using radio-labeled fragments spanning the entire ISRE. Nuclear extracts prepared from each of the three cell lines all generated one band with the use of labeled ISRE probes (marked as band A, Fig. 3A). This band could be completely abolished by adding an excess of unlabeled ISRE fragments (Fig. 3B, lane 3), whereas an excess of unlabeled irrelevant OCT-1 fragments did not have the same effect (Fig. 3B, lane 2). Furthermore, the complex formation was inhibited by treating the nuclear extracts with an anti-IRF-1 antibody but not with an anti-IRF-2 antibody, thus suggesting that IRF-1 bound to the ISRE element of the HLA-A gene (Fig. 3C). As shown in Fig. 3A, the binding of nuclear IRF-1 to the ISRE element in QGY7701 and BEL7402 cells was stronger than that in BEL7405 cells. Consistent with this finding, the total IRF-1 proteins were increased in QGY7701 and BEL7402 cells compared to BEL7405, and paralleled the increased HLA-A expression in these cells (Fig. 3D). These results suggested that augmented binding of nuclear IRF-1 to ISRE was due to the enhanced expression of the IRF-1 gene in QGY7701 and BEL7402 cells.

To further test this possibility, we overexpressed the wild type IRF-1 gene in BEL 7405 cells. Using western blot analysis and flow cytometry, we found that this could partially rescue HLA-A heavy chain and surface HLA-A expression [Fig. 3E and 3F, MFI (Mean fluorescence intensity): BEL7405/mock vs. BEL7405/IRF-1: 45.12 vs. 57.03]. In contrast, transfection with IRF-2, previously described as a transcription repressor of IRF-1, or transfection with a dominant negative form of IRF-1 (without transactivation domain) could lower HLA-A expression in BEL7405 cells (Fig. 3G). Taken together, these data suggested that increased IRF-1 could result in higher HLA-A expression in liver cancer cells.

3.4. P65 regulated HLA-A promoter activity through enhancer A

Total nuclear extracts from the three cell lines generated several bands when incubated with the radiolabeled enhancer A probe, but only band B was weakened in response to competitive inhibition by an excess of cold enhancer A probe in a dose dependent manner (Fig. 4A, lane 1 to lane 3). Using the p65 antibody, band B could be supershifted (Fig. 4A, lane 4). Binding of nuclear proteins to the labeled enhancer A probe resulted in much stronger band B's in QGY7701 and BEL7402 extracts than in BEL7405 extracts, suggesting that more nuclear p65 was bound to the enhancer A element (Fig. 4A, lane 5 to lane 7). Although the total amount of p65 remained almost the same, we observed increased p65 expression in the nuclear extracts of QGY7701 and BEL7402 cells compared to BEL7405 cells by Western analysis. In parallel with this, I κ B, the inhibitor of p65 nuclear translocation and transcriptional activity, was decreased in QGY7701 and BEL7402 cells compared to BEL7405 cell (Fig. 4B). Furthermore, overexpression of exogenous p65 was found to strongly enhance the expression of HLA-A heavy chain in BEL7405 cells (Fig. 4C). This upregulation of surface HLA-A was confirmed by flow cytometry (Fig. 4D, MFI: BEL7405/mock vs. BEL7405/p65: 45.12 vs. 100.07). In contrast, HLA-A expression was

sharply decreased in BEL7405 by transfection of wild type I κ B, or by transfection of an I κ B mutant plasmid, which degrades slowly in the cell cytoplasm (Fig. 4E). These findings suggested that increased p65 activity was another important factor involved in the enhanced HLA-A expression in liver cancer cells.

3.5. Synergistical action of IRF-1 and p65 in HLA-A induction

Cotransfection of IRF-1 and p65 led to an obvious increase in HLA-A promoter activity (Fig. 5A) and surface HLA-A expression, higher than the level of expression achieved by transfection with IRF-1 or p65 alone (Fig. 5B, MFI: BEL7405/mock vs. BEL7405/IRF-1 vs. BEL7405/p65 vs. BEL7405/IRF-1+p65: 45.12 vs. 57.03 vs. 100.07 vs. 356.06).

3.6. Upregulation of HLA-A expression in liver cancer tissues was associated with IRF-1 upregulation and activation of p65

To determine the relationship of the upregulated expression of HLA-A with IRF-1 and p65, we evaluated their expression in the same 21 specimens of fresh liver cancer tissue by western blot. The expression of IRF-1 was correlated with the expression of HLA-A heavy chain, with 11 of the 21 tissue samples showing both increased HLA-A and IRF-1 expression ($\chi^2 = 7.5$, $p = 0.0062$, Table 2, Representative data in Fig. 6A). Although there was no statistically significant correlation between the expression of p65 and HLA-A heavy chain, p65 expression was apparently higher in tumor nuclear extracts compared with non-tumor nuclear extracts in 10 of the 14 tissues (71%) with HLA-A upregulation (Representative data in Fig. 6B). Among them, 8 of the 10 samples demonstrated higher IRF-1 expression, increased p65 nuclear expression, and HLA-A upregulation at the same time (Table 2).

4. Discussion

Hepatocellular carcinoma is one of the most common malignancies in East Asian countries. About 11,000 Chinese people died of HCC annually, making up approximately half of the total numbers of the world population who died of HCC in the same year (Huang et al., 2002). Although there are various modalities of treatment, the prognosis of HCC is still poor. New therapeutic strategies to improve the prognosis of patients with HCC are urgently required. In recent years, studies of CTL vaccines against HCC have become the new frontier of HCC immunotherapy and this novel therapy depends on HLA class I expression. For example, the killing activity of mouse AFP specific CTL to mouse HCC cell lines Hepa1-6, which express few MHC class antigens, is much lower than that of MHC class antigens positive EL4 cells (Vollmer et al., 1999). Thus, whether a particular case of HCC is suitable for treatment with CTL vaccine therapy depends to a great extent on the HLA class antigen expression status of HCC cells.

Based on a small-scale investigation, we previously reported that most liver cancer cells in HCC tissues have strong HLA class I antigen expression, especially at the HLA-A locus. HCC cell lines established from HCC tissues of Chinese patients showed different basal levels of HLA class I expression. For example, upregulated expression of HLA class I antigen in QGY7701 and BEL7402, as well as other HCC cell lines such as Alexander, HepG2, QGY7703, and SMMC7721 has been confirmed in this study and others (Chen et al., 2005). However, the cause of this phenomenon and its immunobiological implications remains to be explained. In this larger scale investigation, we showed for the first time that HLA-A expression was upregulated in 54% of HCC patients. Among several molecules in the antigen processing pathway, enhanced expression of the HLA-A heavy chain was more obvious than those of other molecules (62%). This stimulated us to further investigate the mechanism involved in the increased HLA-A heavy chain expression in liver cancer.

Transcription of the HLA-A gene is regulated by both tissue specific (basal) and cytokine-mediated (active) mechanisms. Gobin and Keijsers (1998) reported that three major regulatory promoter elements, Enhancer A, ISRE, and the SXY module mediated different routes of HLA-A gene transcription. In all the cell lines we investigated, the sequences of all these elements were conserved (data not shown), while their transcriptional activities were different, with enhancer A and ISRE showing increased activities in cells with high HLA-A expression. Enhancer A and the ISRE are the binding sites for nuclear transcription factors of the NF- κ B/Rel family and IRF family, respectively. The NF- κ B/Rel family of transcription factors is comprised of at least 5 members. Among them, the p50/p65 dimer, also termed NF- κ B, with p65 possessing potent transcriptional activity and p50 serving as an ancillary factor, is the most abundant (Molinero et al., 2004). In this study, we were able to demonstrate that the altered amount of endogenous p65 binding to enhancer A was one of the mechanisms by which HLA-A expression was changed in liver cell lines. Other members of NF- κ B/Rel family, although not identified in this study, may also have an ancillary function. We confirmed this hypothesis by increasing and decreasing the amount of p65 in the nuclei of these cell lines. The tight relationship between the amount of nuclear p65 and HLA-A heavy chain expression demonstrated the important transcriptional function of p65 in the regulation of HLA-A expression in liver cancer cells. On the other hand, the ISRE element could be bound by a series of transcription factors that regulate gene transcription in a positive (bound by IRF-1) or negative (bound by IRF-2 or ICSBP) manner (Meraro et al., 2002). Although we could not demonstrate the supershift band in this study using IRF-1 antibodies, we believe that the antibody may disrupt the protein:DNA interaction, resulting in the loss of the characteristic shift. Therefore, we found only IRF-1 binding to the ISRE element of the HLA-A promoter in these cell lines. The relationship between the amount of IRF-1 and HLA-A heavy chain expression also demonstrated the function of the transcription factor IRF-1 in the regulation of HLA-A expression in liver cancer.

After comparing the induction of HLA-A expression and surface HLA-A complex formation by p65 and IRF-1, we came to the conclusion that p65 contributes more to HLA-A expression in HCC cells compared to IRF-1. This is in keeping with other studies suggesting that IRF-1 has only weak enhancer activity on its own (Girdlestone, 2000). Previous reports also described a synergistic interaction between NF- κ B and IRF-1 to induce HLA and other gene expressions (Naschberger et al., 2004). In our present study, we observed that the combined activity of the enhancer A and ISRE was greater than the individual activity of either of the two. In conclusion, both transcriptional factors IRF-1 and p65 contributed to the constitutive HLA-A upregulation in liver cancer, with p65 demonstrating stronger transcriptional activity than IRF-1. Furthermore, they acted synergistically to induce HLA-A transcription in liver cancer.

HCC is found in conjunction with the effect of chronic inflammation and cirrhosis, accompanied by a regenerative process. This provides a common pathway to the development of HCC from liver tissue chronically infected with HBV or HCV. HCV-infected cells can recognize the double strand virus RNA by surface Toll-like receptors, which in turn trigger activation of nuclear factor κ B, production of type-1 interferons, and finally induction of HLA class I expression in these cells and surrounding tissues (Dolganiuc et al., 2007). How the hepatic cells react to HBV, a double strand DNA virus, is not clear compared to HCV infection. Although it is reported that an established cell model transfected with whole HBV gene can induce stronger expression of HLA-A, -B, -C, and MICA/B on hepatic cells (Chen et al., 2007), others have demonstrated unchanged or even decreased HLA class I expression upon HBV gene transfection (Chen et al., 2006). This study did not reveal the direct impact of HBV infection on HLA class I expression. First, there was no relationship between HBV infection and increase in HLA-A expression in 165

patients investigated, although the majority of the cases (143/165) showed positive expression of HBsAg. Moreover, none of the liver cell lines showed expression of HBsAg or the HBV X gene, indicating no HBV existed in cell (supplementary Fig. 1 and Fig. 2). Despite the lack of HBV interfering, the constitutive HLA-A expression varied among the cell lines, with higher expression of HLA-A in QGY7701 and BEL7402 than in BEL7405. Whether HBV infection is one of the reasons for increased HLA class I expression in hepatocarcinogenesis will be discovered if we can understand the complex network of cellular antiviral process and virus–host interaction of HBV infection in the future.

It is now clear that NK cells can perform ‘missing self’ recognition, that is, the ability to eliminate cells which fail to express certain critical HLA class I molecules. Natural killer (NK) cells are abundant in the normal liver, accounting for approximately one-third of intrahepatic lymphocytes (Herzer et al., 2003). Increased expression of HLA class I antigen can explain how liver cancer cells are able to evade an innate immune response. On the other hand, HBV-specific cytotoxic T cell therapy can be a suitable and practical therapy for more than half of the HCC patients with high HLA class I expression. Selection of patients with high HLA class I expression is a crucial step towards successful immunotherapy. Since NF- κ B/IRF1 mediates the response to tumor necrosis factors and type-1 interferons, treatment with both of them in the remaining low HLA class I expression group would be beneficial for rescuing HLA expression and clinical T cell based immunotherapy in liver cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. HLA-A expression was elevated in liver cancer. (A) Typical cell surface expression of HLA-A antigen in paraffin embedded liver cancer tissue (left panel) with negative expression in its normal compartment (right panel, positive staining of infiltrate lymphocyte and endothelial cells, Immunohistochemistry, 200x). (B) Increased expression of HLA-A complex in fresh liver cancer tissue (left panel) with negative expression in its normal compartment (right panel, positive staining of infiltrate lymphocyte and endothelial cells, Immunohistochemistry, 200x). (C) The expression of several molecules in the antigen processing pathway was detected by RT-PCR in liver cancer tissue (T) compared to its noncancerous part (N), with increased expression of HLA-A heavy chain in all the 3 samples.

Fig. 2.

The ISRE and enhancer A were important elements for the constitutive HLA-A promoter activity in liver cancer cell lines. (A) Schematic illustration of constructs used for HLA-A heavy chain promoter-luciferase reporter assay. AP: HLA-A heavy chain promoter (from -217 bp to the transcriptional initiation site); AP-ISRE del: HLA-A heavy chain promoter without ISRE; AP1: HLA-A heavy chain promoter without enhancer A; AP2: HLA-A heavy chain promoter without enhancer A and ISRE; AP3: HLA-A heavy chain promoter without enhancer A, ISRE, site α and enhancer B. All of them were constructed into pGL3-Basic luciferase reporter plasmid. (B) Increased HLA-A expression in HCC cell lines QGY7701 and BEL7402 compared to BEL7405. Total cellular RNA from the three cells were extracted and subjected to Northern blot analyses. The expression of the β -actin gene was served as the internal control. (C) Luciferase activity of different promoter-reporter plasmids in the three HCC cell lines. For each cell line, mean luciferase activity was calculated from three independent experiments and shown with the standard error. (D) Comparison of the activity of enhancer A and ISRE in different HCC cell lines. The activity of enhancer A, ISRE, or enhancer A + ISRE was calculated by dividing the mean luciferase activity of each reporter construct with the element by that of the comparable construct without the element. Both of the enhancer A and ISRE activity were obviously increased in QGY7701 and BEL7402 cells compared to BEL7405. Besides that, the combined transcriptional activity of enhancer A and ISRE was more than that of the individual one.

Fig. 3.

Transcription factor IRF-1 regulated HLA-A promoter activity through binding to ISRE in liver cancer cell lines. (A) The intensity of band A was increased in QGY7701 and BEL7402 cells than in BEL7405 cells. Band A was formed by incubating labeled ISRE probes with nuclear extracts of QGY7701, BEL7402 or BEL7405. Incubating labeled ISRE probes with nuclear extracts of IFN- γ treated BEL7405 was used as the positive control (lane 2). (B) Band A could be competed by cold ISRE probe. Nuclear extracts of QGY7701 were incubated with labeled ISRE probes either in the presence of 200-fold excess of unlabeled OCT-1 fragments (lane 2) or of unlabeled IRF-1 fragments (lane 3). (C) Nuclear extracts of QGY7701 were incubated with labeled ISRE probes (lane 2) and band A was decreased in the presence of anti-IRF-1 antibody (lane 1), but not of anti-IRF-2 antibody (lane 3). (D) The expression of HLA-A heavy chain and IRF-1 protein was detected in HCC cell lines by Western analysis. IRF-1 and HLA-A were both increased in QGY7701 and BEL7402 cells compare to BEL7405 cells. The amount of HLA-A was calculated as the ratio of the density of the HLA-A band to the density of the β -actin band by using Software

Image 7. Other bands in the following figures were calculated by the same way except the result of Western blot analysis of nuclear protein. (E) Elevated expression of HLA-A heavy chain was detected by Western analysis after transfection of full length of IRF-1 gene into BEL7405 cells. The expression of HLA-A heavy chain in QGY7701 cells was added as the positive control. (F) Increased expression of surface HLA-A complex in BEL7405 cells transfected with IRF-1 was demonstrated by indirect flow cytometry analysis with antibody LGIII-147.4.1. The gray area represented background staining of the cells with the isotype-matched control antibody. The dotted line and the thin solid line represented HLA-A expression of BEL7405 cells transfected with an empty plasmid and with IRF-1, respectively. (G) Detected by Western analysis, the expression of HLA-A heavy chain was decreased after transfection of dominant negative form of IRF-1 or IRF-2 in BEL7405 cell.

Fig. 4.

Transcription factors p65 binding to enhancer A regulated HLA-A promoter activity in liver cancer cell lines. (A) P65 binding to enhancer A was increased in QGY7701 and BEL7402 cells compared to BEL7405. Nuclear extracts of QGY7701 was incubated with labeled enhancer A probes (lane 5, band B). In the presence of 50-, 100-, or 200-fold excess of unlabeled enhancer A probes (lanes 1, 2, and 3), band B was weakened in response to competitive inhibition in a dose depended manner. Band B was supershift by anti-p65 mAbs (lane 4) and the intensity of band B was increased in the nuclear extracts of QGY7701 and BEL7402 compared to BEL7405 (lanes 5, 6, and 7). (B) The expression of total p65, IκB, and nuclear p65 was examined in HCC cell lines by Western blot. The expression of p65 was increased only in the nuclear extracts of QGY7701 and BEL7402 cells compared to that of BEL7405 cells (The number below each band was calculated as the ratio of the density of the p65 band in the BEL7402 cells and BEL7405 cells to that in the QGY7701 cells). In contrast, the expression of IκB was decreased in QGY7701 and BEL7402 cells. (C) Detected by Western analysis, HLA-A heavy chain proteins were strongly induced by transfection of p65 expression plasmid. (D) Increased cell surface expression of HLA-A complex in BEL7405 upon p65 transfection was detected by flow cytometry analysis with antibody LGIII-147.4.1. The gray area represented background staining of the cells with the control antibody. The dotted line and the thin solid line represented HLA-A expression of BEL7405 cells transfected with an empty plasmid and with p65, respectively. (E) The expression of HLA-A protein was specifically decreased in BEL7405 by transfection of IκB or of an IκB mutant plasmid, which degrades slowly in cell cytoplasm than wild type IκB.

Fig. 5.

Synergistic induction of HLA-A expression by IRF-1 and p65. (A) Detected by luciferase assay, p65 transactivated HLA-A promoter–reporter (from –217 bp to the transcription initiation site) more strongly than IRF-1 in BEL7405 cell, while the highest induction was seen when the cell was co-transfected with IRF-1 and p65. (B) Simultaneously transfection of the two factors led to an obviously increased HLA-A complex expression over the level of expression achieved by transfection with IRF-1 or p65 separately. The gray area showed background staining of the cells with the control antibody. HLA-A expression in BEL7405 cells transfected with empty plasmid was represented by dotted line; transfected with IRF-1, thin solid line; transfected with p65, black line; transfected with both IRF-1 and p65, shallow gray area.

Fig. 6. Enhanced HLA-A expression in liver cancer tissues was associated with IRF-1 upregulation and activation of NF-KB. (A) Upregulation of IRF-1 and increased HLA-A expression in the same HCC tissue (number 10). Beta-actin was included as an internal control. The expression of IRF-1 and HLA-A in QGY7701 cells was used as a positive control (pc). (B) Increased expression of nuclear p65 with enhanced expression of HLA-A heavy chain in the same HCC tissue (number 21).

Table 1

The expression of molecules in antigen processing pathway in liver cancer.

HLA antigen processing pathway molecules					
	HLA-A	β2m	TAP1	TAP2	Tapasin
Upregulation	13(62%)	9(43%)	10(50%)	12(57%)	8(38%)
Consensus expression	4	10	6	4	9
Downregulation	4	7	5	5	4
Total	21	21	21	21	21

RT-PCR was used to compare the expression of HLA antigen processing pathway molecules in tumor and normal tissues. Expression of each component of the pathway was quantified by Software Image 7 as the ratio of the molecule's band density to that of β-actin. Then, the ratio of the expression of each component in the cancerous region to each in the normal region was taken. The ratios were classified into three groups: upregulated (ratio >2), downregulated (ratio <0.5), or consensus (ratio 0.5–2).

Table 2

The expression of HLA-A, IRF-1 and nuclear p65 in liver cancer tissues.

	HLA-A↑ IRF-1↑		IRF-1↓/=		HLA-A↓/= IRF-1↑		IRF-1↓/=	Total
Nuclear p65↑	8	2	0	0	3	3	3	13
Nuclear p65↓/=	3	1	1	1	3	3	3	8
Total	11	3	1	1	6	6	6	21

The expression of HLA molecules in tumor tissue were compared to its normal compartment by Western blot. Band intensities were calculated as the same way described in Fig. 3D. “↑” means increased molecular expression in cancerous part compared to its normal part (ratio >2), “↓/=” means decreased or equal molecular expression in tumor tissues compared to its normal compartment (ratio <=2).