Sucrase-Isomaltase Gene Expression Is Inhibited by Mutant Hepatocyte Nuclear Factor (HNF)-1α and Mutant HNF-1β in Caco-2 Cells

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Summary Hepatocyte nuclear factor (HNF)-1α and HNF-1β are concerned in sucrase-isomaltase (SI) gene expression, and directly bind two sites (SIF2, SIF3) of the promoter of the SI gene. However, it is not completely clear that HNF-1α and HNF-1β play a role in regulation of SI gene expression. To clarify mechanisms of SI gene expression regulated by HNF-1α and HNF-1β, we established four stable cell lines based on enterocyte-like cell line Caco-2, in which wild HNF-1α or wild HNF-1β, or else mutant HNF-1αT539fsdelC or mutant HNF-1βR177X was overexpressed. In the HNF-1αT539fsdelC cells and HNF-1βR177X cells, but not in the wild HNF-1α cells and wild HNF-1β cells, SI gene expression and enzyme activity were significantly diminished compared with that in Caco-2 cells. Moreover, to clarify whether or not stable cell differentiation was influenced by overexpression of these transgenes, alkaline phosphatase (ALP) gene expression and enzyme activity were measured. There were no changes in ALP gene expression or enzyme activity in these cells. These observations suggest that mutant HNF-1αT539fsdelC and mutant HNF-1βR177X inhibits SI gene at the transcriptional level, resulting in decreased SI enzyme activity in Caco-2 cells. We propose that both HNF-1α and HNF-1β would contribute to constitutive expression of the SI gene in the differentiated state in Caco-2 cells.

Key Words SI, HNF-1α, HNF-1β, ALP, Caco-2

Sucrase and isomaltase form a complex enzyme, which is a major component of α-glucosidase and is locally expressed in epithelial intestinal cells (1). The SI complex is synthesized as a single precursor polypeptide, which matures by being split into sucrase and isomaltase subunits by a pancreatic protease (2). The enzyme is essential for the digestion of sucrose and for the terminal digestion of starch, and plays an important role in digestion and absorption of ingested carbohydrates (3).

SI gene expression and enzyme activity are changed by nutritional conditions and several diseases. There is evidence from studies using experimental animals that SI gene expression and enzyme activity are enhanced by dietary intake, and, conversely, are reduced by fasting (4, 5). In diabetic patients and model animals, SI gene expression is augmented, resulting in incremental SI enzyme activity (6–8). Enhanced SI enzyme activity may cause postprandial hyperglycemia (PPG), and aggravate diabetic conditions. Therefore, α-glucosidase inhibitor is very useful for treatment and prevention of type 2 diabetes mellitus (9).

Recently, several studies have been conducted in regard to the regulation mechanism of SI gene expression (10–12). It is reported that hepatocyte nuclear factor (HNF)-1α and HNF-1β are concerned in SI gene expression, directly binding two sites (SIF2, SIF3) of the promoter of the SI gene (11). However, it is not completely clear that HNF-1α and HNF-1β play a role in the regulation of SI gene expression. HNF-1α and HNF-1β are found in the small intestine, liver, pancreas and kidneys (13). HNF-1α and HNF-1β are each composed of three functional domains: the dimerization domain, the DNA-binding domain and the transcriptional domain. HNF-1α and/or HNF-1β binding DNA are present as heterodimers and homodimers through the dimerization domain (14). Mutant HNF-1α and mutant HNF-1β lead to synthesis of truncated proteins with simple loss of function, or with dominant-negative
effects (15–17). In a previous luciferase assay study, we found that mutant HNF-1αT539fsdelC and mutant HNF-1R177X have a dominant-negative effect on the wild HNF-1α and wild HNF-1β (18).

The Caco-2 cell is an optical model for studying SI gene expression and enzyme activity in vitro. The SI gene is spontaneously expressed in the differentiated state in Caco-2 cells, but is hardly found at all during the preconfluent and the early confluent states (19). Therefore, it is impossible to analyze changes in the level of endogenous SI gene expression and activity by a transient study using undifferentiated Caco-2 cells. In the present study, we established four stable cell lines based on the enterocyte-like cell line Caco-2, in which wild HNF-1α or wild HNF-1β, or else respective dominant negative mutant HNF-1αT539fsdelC or HNF-1R177X, was expressed. We clarified that HNF-1α and HNF-1β play a role in the regulation of SI gene expression and activity by examining SI gene expression and enzyme activity in stable cells in the differentiated state.

MATERIALS AND METHODS

Vector constructs of wild or mutant HNF-1α and wild or mutant HNF-1β. Mutant HNF-1αT539fsdelC and mutant HNF-1R177X have mutant HNF-1αT539fsdelC generating a protein of 539 normal residues at the N-terminus, followed by a protein of 119 abnormal amino acids. Mutant HNF-1R177X generates a truncated protein of 176 amino acids with the normal N-terminus. In our previous study, these proteins were shown to have a dominant-negative effect (18). In the present study, a part of the HNF-1α (nucleotides: −781 to +6 bp) or HNF-1β (nucleotides: −832 to +5 bp) promoter site was combined with wild HNF-1α cDNA or wild HNF-1β cDNA, mutant HNF-1αT539fsdelC cDNA or mutant HNF-1R177X cDNA, to imitate the natural expression patterns of HNF-1α and HNF-1β. These cDNAs were subcloned into plRESneo2 vectors (Clontech, USA), which were, in turn, transfected into Caco-2 cells. Caco-2 cells in which wild HNF-1α cDNA, mutant HNF-1αT539fsdelC cDNA, wild HNF-1β cDNA, and mutant HNF-1αT539fsdelC cDNA were stably expressed were considered stable cell lines: wild HNF-1α cells, HNF-1αT539fsdelC cells, and wild HNF-1β cells, HNF-1R177X cells, respectively.

Cell culture. Caco-2 and stable cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 1.0 g/L d-glucose, 20% fetal calf serum, 100 U/mL penicillin, and 100 mg/L streptomycin in 10 cm dishes (Iwaki, Japan), in 5% CO2 at 37°C. The medium was changed every 48 h. Cells in the 23 passages were seeded at a density of 15,000 cells per dish, and became confluent after 3 d. These cells were collected on the 1st and 10th confluent days for analysis of gene expression and activity.

Transfection and stable cell selection. Caco-2 cells were plated in 60 mm dishes (Iwaki) at a density of 8,000 cells per dish. Transfection of Caco-2 cells with wild HNF-1α or wild HNF-1β and mutant HNF-1αT539fsdelC or mutant HNF-1R177X expression vector was performed using LipofectAMINE Reagent (Invitrogen, USA). A mixture of each expression vector DNA (1.0 μg) and 12.0 μL LipofectAMINE reagent in 250 μL medium (FCS and antibody free) was added to each dish. The dishes were incubated in 5% CO2 at 37°C, and the mixture was replaced with complete medium after 3 h. All cells were harvested and subcultured into fresh complete medium 1 d after the start of transfection, and 1.0 mg/mL G418 was added after 2 d to select for expression of the resistance gene. Individual clones appeared after approximately 3 wk, and were trypsinized individually with cloning rings. Selected clones were maintained in 0.5 mg/mL G418.

Genomic DNA and RNA isolation, and RT-PCR. Genomic DNA and total RNA were isolated from the stable cells and Caco-2 cells. First-strand cDNA synthesis reactions were performed using Superscript-II enzyme (Invitrogen). Specific mRNA levels were quantitated by the real-time PCR method, using the DNA Engine Opticon2 System and DyNaMo HS SYBR Green qPCR kit (MJ Research). In HNF-1αT539fsdelC and HNF-1R177X cells, transfected mutant or endogenous wild HNF-1α and wild HNF-1β gene expression was determined by the allele-specific primer method (20, 21).

| Gene name (Accession No.) | Forward (5′→3′) | Reverse (5′→3′) | PCR product (bp) |
|--------------------------|----------------|----------------|-----------------|
| SL (X6579)               | CATCCTACATGTCAAGAAGCA | GCTTGTTAAGGTGTTGCTGTTT | 196 |
| ALP (M15694)             | TCAGTCATCTCCAACACGGG | TTAGATGAGGTACAGACTGG | 225 |
| HNF-1α (M57732)          | TACACCTGTGAAGTTCGCCA | CACTTGAAACGGTTCCTCCG | 131 |
| HNF-1β (X58840)          | CCACAATCTCCTCCTACCCTATG | GGCTGTTGAATATTTCGTAAGT | 89 |
| HNF-1αT539fsdelC (M57732)| CTCCGACAGTAACTGCTC | AGGTTGAGACTCTACAGT | 82 |
| HNF-1R177X (X58840)      | GTAGATCTGAGAAGCCTAC | AACAGGCTCATCTGACT | 99 |
| GAPDH (M33197)           | GAAGGTTGACAGTGCGGTC | GAAGATGGTATGTCGATTC | 226 |

Primers of SL, ALP, HNF-1α, HNF-1β, and GAPDH were used to quantify relevant gene expression. Primers of HNF-1αWT and HNF-1βWT, HNF-1αT539fsdelC and HNF-1R177X were used to detect exogenous wild HNF-1α and wild HNF-1β, HNF-1αT539fsdelC and HNF-1R177X transgenese at the genomic DNA and mRNA levels in the stable cells. The first lower case letter at 3′ terminus of the primer is the specific mutant base, and the second lower case letter is the artificially mismatched base.
The primers are shown in Table 1. Respective gene expression was normalized by the GAPDH gene.

**Enzyme activity.** The cells were harvested after being washed three times in ice-cold PBS (Mg²⁺ and Ca²⁺ free). Enzyme activity was determined after partial purification of the brush border membranes according to the technique of Schmitz et al. (22). SI and ALP activities were assayed according to the previous method (23, 24). SI and ALP enzyme assay units were defined as nmol glucose mg⁻¹ cellular protein per minute and μmol p-nitrophenol mg⁻¹ cellular protein per minute, respectively. Proteins were determined by the method of Lowry et al. (25).

**Statistical analysis.** Statistical evaluation was performed with Student’s t-test using the Statview Statistical Package (SAS Institute, Cary, NC). All data are shown as mean±SE, and statistical significance is defined as p<0.05.

**RESULTS**

**Detection of exogenous transgenes in genomic DNA and mRNA levels**

To clarify whether exogenous wild or mutant HNF-1α and wild or mutant HNF-1β genes were expressed in stable cells, we determined whether these exogenous transgenes were present at the genomic DNA and mRNA levels. As a result, we detected these exogenous transgenes both at the genomic DNA level and at the mRNA level (Fig. 1A, B). Due to the fact that the primers spanned the exon-exon boundary of the genes, endogenous HNF-1α and HNF-1β genes from the genomic DNA of Caco-2 cells were not detected (Fig. 1A, B). Moreover, because HNF-1α and HNF-1β gene were naturally expressed at the mRNA level in Caco-2 cells, we also detected their expression in all stable cells (Fig. 1A, B). Although we found that three mutant HNF-1α (R271G, P379fsdelCT, T539fsdelC) and one mutant HNF-1βR177X have a dominant-negative effect in the previous transient study (18), the stable transgene expression of mutant HNF-1αT539fsdelC and mutant HNF-1βR177X was particularly noticeable in the present study.

**Expression of wild or mutant HNF-1α and wild or mutant HNF-1β in stable cells and Caco-2 cells**

It has been reported that HNF-1α and HNF-1β gene expressions change with Caco-2 cell differentiation (26). Therefore, we measured HNF-1α and HNF-1β expression in Caco-2 cells (as a control), and in stable

![Fig. 1. Detection of exogenous wild HNF-1α and mutant HNF-1αT539fsdelC (A) and wild HNF-1β and mutant HNF-1βR177X (B) transgenes at the genomic DNA and mRNA level. Wild and mutant HNF-1α or wild and mutant HNF-1β constructs were used as controls.](image-url)
cells. HNF-1α expression increased (Fig. 2A), whereas HNF-1β expression decreased (Fig. 3A) with differentiation of these cells. In HNF-1αT539fsdelC and HNF-1αR177X cells, a mutant transgene and endogenous wild gene are coincidentally expressed; this fact was used to determine the presence of mutant transgene and endogenous wild gene expression. The expression of the mutant transgene was greater than that of the endogenous wild gene (Fig. 2B and Fig. 3B). These results suggest that high expression of HNF-1αT539fsdelC and HNF-1βR177X should play a dominant negative effect in respective stable cells.

Fig. 2. A: HNF-1α gene expression in cells, except for HNF-1αT539fsdelC cells. White bar: on the 1st confluent day; black bar: on the 10th confluent day. *p<0.05 compared with that on the 1st confluent day. HNF-1α gene expression in these cells was normalized by that in Caco-2 cells on the 1st confluent day, which is defined as 1 unit. B: Wild HNF-1α and mutant HNF-1αT539fsdelC gene expression in HNF-1αT539fsdelC cells. White bar: on the 1st confluent day; black bar: on the 10th confluent day. *p<0.05 compared with that on the 1st confluent day. Wild and mutant HNF-1α expression was normalized by wild HNF-1α gene expression on the 1st confluent day, which is defined as 1 unit.

Fig. 3. A: HNF-1β gene expression in cells, except for HNF-1βR177X cells. White bar: on the 1st confluent day; black bar: on the 10th confluent day. *p<0.05 compared with that on the 1st confluent day. HNF-1β gene expression in these cells was normalized by that in Caco-2 cells on the 1st confluent day, which is defined as 1 unit. B: Wild HNF-1β and mutant HNF-1βR177X gene expression in the HNF-1βR177X cells. White bar: on the 1st confluent day; black bar: on the 10th confluent day. *p<0.01 compared with that on the corresponding day. Wild and mutant HNF-1β expression was normalized by wild HNF-1β gene expression on the 1st confluent day, which is defined as 1 unit.

**SI gene expression and enzyme activity in stable cells and Caco-2 cells**

It has been reported that SI enzyme activity closely parallels its gene expression level (27). On the 1st confluent day, there were no differences between Caco-2 cells and stable cells as to SI gene expression or enzyme activity (Fig. 4). However, SI gene expression and enzyme activity were significantly lower in HNF-1αT539fsdelC and HNF-1βR177X cells than in Caco-2 cells on the 10th confluent day (Fig. 4). No differences in SI gene expression or enzyme activity were observed among wild HNF-1α, wild HNF-1β cells, or Caco-2 cells (Fig. 4).
To elucidate whether overexpression of wild HNF-1α and wild HNF-1β, or mutant HNF-1αT539fsdelC and mutant HNF-1βR177X, affects cells differentiation, we measured alkaline phosphatase (ALP) gene expression and enzyme activity, which is a marker of Caco-2 cell differentiation (28), and which has not been reported to be related to HNF-1α and HNF-1β. Our results showed that there were no changes in these cells (Fig. 5). These results suggest that overexpression of wild or mutant HNF-1α and wild or mutant HNF-1β did not impair Caco-2 cell differentiation.

**DISCUSSION**

In this study, we investigated whether mutant HNF-1α and mutant HNF-1β affect SI gene expression and enzyme activity in the enterocyte-like cell line Caco-2. This would help us to elucidate the mechanisms of SI gene expression regulated by HNF-1α and HNF-1β. The Caco-2 cell is an optical model for studying SI gene expression and enzyme activity in vitro. SI and HNF-1α and HNF-1β genes are found to be expressed in the differentiated state in Caco-2 cells (11). However, it is not completely clear that HNF-1α and HNF-1β play a role in the regulation of SI gene expression.

In the present study, HNF-1α expression increased (Fig. 2A), whereas HNF-1β expression decreased (Fig. 3A), with Caco-2 cell differentiation; simultaneously, a strong induction of SI gene expression was observed in Caco-2 cells (Fig. 4A). It seems that HNF-1α plays a major role in the regulation of SI gene expression. In stable cells, high levels of wild HNF-1α and wild HNF-1β, as well as mutant HNF-1αT539fsdelC and mutant...
HNF-1βR177X, were expressed (Figs. 2 and 3). Since a part of HNF-1α or HNF-1β gene promoter was used in these expression vectors, the expression patterns of these genes were similar to those in Caco-2 cells (Figs. 2 and 3). Although our previous study had shown that both wild HNF-1α and wild HNF-1β are capable of increasing SI-luciferase reporter gene expression (18), there were, surprisingly, no differences in SI gene expression or enzyme activity in wild HNF-1α cells or wild HNF-1β cells, as compared to Caco-2 cells (Fig. 4). SI gene was little expressed at the beginning of differentiation, although expression increased in Caco-2 cells on confluent days (Fig. 4). Using preconfluent undifferentiated Caco-2 cells, we found that endogenous SI gene expression and activity were not changed by transient overexpression of wild or mutant HNF-1α and wild or mutant HNF-1β (data not shown) (18). In Caco-2 cells in which the SI promoter-human growth factor reporter gene was stably overexpressed, reporter gene expression was not changed on the 1st confluent day, irrespective of whether a wild or mutant site was located in the SI promoter (29). It is likely that SI gene expression is also closely associated with the differentiation state of Caco-2 cells. In the preceding transient study, wild HNF-1α and wild HNF-1β that were excessively expressed did not have an additional transcriptional effect on SI-luciferase reporter gene expression (18). Furthermore, in transgenic mice, and in stable cells based on INS-1 cells, target gene expression is not affected by overexpression of wild HNF-1α (30, 31). These facts indicate that SI gene expression in the Caco-2 cells is not merely parallel to the amount of HNF-1α or HNF-1β, but is probably also associated with the differentiation state of the cell and other unknown factors. Perhaps that is one reason why the high level expression of wild HNF-1α and wild HNF-1β did not promote SI gene expression on the 1st and 10th confluent day.

On the other hand, in HNF-1αT539fsdelC and HNF-1βR177X cells, although SI gene expression and enzyme activity were somewhat increased, they were considerably lower than that in Caco-2, wild HNF-1α, and wild HNF-1β cells on the 10th confluent day (Fig. 4). In a previous transient study of co-expressed wild HNF-1α and mutant HNF-1αT539fsdelC, or wild HNF-1β and mutant HNF-1βR177X, SI-luciferase reporter gene expression was hardly expressed at all (18). SI gene expression is also reportedly related to other transcription factors, such as Cdx-2 and GATAs, in addition to HNF-1α and HNF-1β, which temporally and spatially regulate SI gene expression (32). These transcription factors probably compensate for effective loss of HNF-1α and HNF-1β, so that SI gene expression and activity are somewhat increased in HNF-1αT539fsdelC and HNF-1βR177X cells. Mutation of HNF-1αT539fsdelC is located in the transcriptional domain, but its dimerization and DNA-binding domain remain normal (33). It has been suggested that HNF-1αT539fsdelC may bind wild HNF-1α or wild HNF-1β via a homodimer or heterodimer, and thus interfere with its normal function. The HNF-1βR177X mutant only has a normal dimerization domain, and lacks both DNA-binding and transcriptional domains (16). It is thought that the dominant negative effect of HNF-1βR177X is more serious than that of HNF-1αT539fsdelC. Interestingly, we found in our previous study that HNF-1βR177X impairs normal function of wild HNF-1β more severely than that of wild HNF-1α (18). SI gene expression and enzyme activity were impaired in HNF-1βR177X cells, perhaps mainly due to interference with the normal function of wild HNF-1β. HNF-1α and HNF-1β, as transcription factors, must be imported into cell nuclei in order to function normally. In previous studies, the nuclear importation of HNF-1α and HNF-1β mutations in the transcriptional domain had been reduced. It has been suggested that there is an essential nuclear localization signal in both mutant sites (16, 17). It seems that HNF-1αT539fsdelC and HNF-1βR177X may impair SI gene expression by attenuating transcription activity and nuclear importation of wild HNF-1β and/or wild HNF-1α.

HNF-1α and HNF-1β have been reported to play an important role in liver cells differentiation. When differentiated hepatoma cell lines are used, expression of transfected HNF-1α may lead to hepatic differentiation (34). Furthermore, SI is known as a marker of Caco-2 cell differentiation. It has been postulated that overexpression of HNF-1αT539fsdelC and HNF-1βR177X also inhibits Caco-2 cell differentiation, resulting in decreased SI gene expression and enzyme activity. To test this hypothesis, we examined expression and enzyme activity of the alkaline phosphatase gene (ALP). ALP is also a marker of Caco-2 cell differentiation (28), and is reportedly not related to HNF-1α or HNF-1β. Our results showed that there was no difference in ALP between stable cells and Caco-2 cells (Fig. 5). It has been suggested that overexpression of mutant HNF-1αT539fsdelC and mutant HNF-1βR177X does not affect Caco-2 cell differentiation. These findings indicate that mutant HNF-1αT539fsdelC and mutant HNF-1βR177X inhibit the SI gene at the transcriptional level, resulting in decreased SI enzyme activity. In the present study, we investigated the roles that HNF-1α and HNF-1β play in SI gene expression and activity by observing stable expression of wild or mutant HNF-1α and wild or mutant HNF-1β in Caco-2 cells. Although, there was no additional increase in SI gene expression and activity in wild HNF-1α and wild HNF-1β cells, we propose that both HNF-1α and HNF-1β would contribute to the constitutive expression of SI gene in differentiated Caco-2 cells. Based on the fact that they were strongly inhibited in the HNF-1αT539fsdelC cells and in the HNF-1βR177X cells. The molecular mechanism of SI gene expression is rather complex, since it is related to other transcription factors, such as Cdx-2 and GATAs, in addition to HNF-1α and HNF-1β (32). Their combined effect is not completely understood, and further detailed study is therefore required.
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