INS-1 베타 세포에서 TNF-α에 의한 포도당 자극 인슐린 분비 저해 기작 연구

배경 및 목적: 체장 소도의 베타 세포는 인슐린을 합성, 분비하여 포도당 자극에 따라 혈당을 조절하는 역할을 한다. 베타 세포에서의 인슐린 분비는 포도당의 유입, 포도당 대사, TCA 회로, 산화적 인산화과정을 거쳐 ATP/ADP 비율이 증가되면 K<sub>ATP</sub> channel이 닫히고 그에 따른 탈분극에 의해 전압 의존적인 Ca<sup>2+</sup> channel이 열려서 Ca<sup>2+</sup> 유입되는 경로와 증가한 [Ca<sup>2+</sup>]i에 의한 인슐린 분비의 효율을 높이는 신호 전달 경로가 생성되어 이루어진다. 그런데 제 2형 당뇨병에서는 이러한 인슐린 분비가 감소되어있다. 포도당 자극 인슐린 분비(GSIS) 저하의 원인은 여러 가지로 생각되지만 비만형 제 2형 당뇨병 환자가 증가되어 있는 Tumor necrosis factor-α (TNF-α)는 베타 세포에서 포도당 자극 인슐린 분비를 감소시키는 하나의 요인으로 생각되어지고 있다. 그러나 그 TNF-α가 어떻게 베타 세포의 포도당 자극 인슐린 분비 저하를 일으키는지 아직 자세히 연구되어 있지 않았다. 그래서 우리는 TNF-α에 의한 포도당 자극 인슐린 분비 저해 기작을 알아보고자 본 연구를 수행하였다.

결 과: 우선 INS-1 베타 세포에 TNF-α를 전처리한 후, 인슐린 분비 저해 효과를 조사하여 보았다. INS-1 베타 세포에서 TNF-α를 농도별, 시간별로 전처리하였을 때, 포도당 자극 인슐린 분비는 농도, 시간에 따라 각각 감소하는 양상을 보였다. 감소한 포도당 자극 인슐린 분비는 TNF-α를 제거하면 정상으로 회복되었다. 에너지 합성 대체 물질인 Leucine와 Glutamine, K<sub>ATP</sub> channel을 닫음으로서 인슐린 분비를 일으키는 것으로 알려져있는 sulfonylurea류 및 imidazolin류에 의한 인슐린 분비 정도도 TNF-α 전처리에 의해서 40~50%까지 감소하는 것으로 나타났다. Ca<sup>2+</sup> channel을 열게하여 인슐린 분비를 일으키는 Bay K8644에 의한 인슐린 분비도 TNF-α 전처리에 의해서 50%까지 감소하였다. 탈분극을 일으켜 인슐린 분비를 일으키는 고농도의 KCl에 의한 인슐린 분비도 약간 감소하는 경향을 나타냈고, K<sub>ATP</sub> channel과 독립적으로 인슐린 분비를 촉진하는 물질인 PMA, forskolin에 의한 인슐린 분비는 TNF-α의
전처리에도 불구하고 저해 받지 않았다. INS-1 베타 세포에 TNF-α를 24시간 동안 처리하였을 때 인슐린 및 GLUT2와 Glucokinase의 mRNA와 단백질 발현량은 감소하지 않았다. 그리고 K\textsubscript{ATP} channel을 닫는데 필요한 ATP 합성량도 변화가 없었다. 이 결과로 TNF-α가 인슐린 생성성 및 포도당 유입, 포도당 대사에 큰 영향을 가져지 않았음을 확인하였다. TNF-α의 전처리는 베타 세포에서 포도당 자극 Ca\textsuperscript{2+} 유입량과 박동성을 감소시켰다. INS-1 세포에서 TNF-α는 단독으로 세포 내 기저 Ca\textsuperscript{2+} 농도를 약간 증가시키는 것으로 나타났다. TNF-α 전처리에 의해 감소된 포도당 자극 인슐린 분비가 L-type Ca\textsuperscript{2+} channel blocker인 Nifedipine과 K\textsubscript{ATP} channel opener인 Diazoxide에 의해서 100% 회복되었다. 이 결과로 TNF-α에 의한 기저 Ca\textsuperscript{2+}농도의 증가가 포도당 자극 Ca\textsuperscript{2+} 유입량의 감소를 일으키는 것으로 생각되고 TNF-α에 의한 기저 Ca\textsuperscript{2+}농도의 증가는 L-type Ca\textsuperscript{2+} channel을 통해 유입되는 것으로 생각된다. 그러나 Ca\textsuperscript{2+}유입량을 조절하는 K\textsubscript{ATP} channel과 L-type Ca\textsuperscript{2+} channel의 mRNA 및 단백질 발현량은 TNF-α 전처리에 의해 영향을 받지 않는 것으로 나타났다. TNF-α에 의해 활성화되는 신호 전달 물질이 포도당 자극 인슐린 분비의 저하와 관련되어있지는 않아보고자 ERK, p38, JNK, NFkB의 활성화가 미치는 영향을 조사하여보았다. TNF-α 전처리에 의해 감소되었던 포도당 자극 인슐린 분비는 NFkB 저해제인 SN50에 의해서 회복되었다. TNF-α에 의한 NFkB의 활성화가 포도당 자극 인슐린 분비를 저해하는데 일부 관여할 것으로 생각된다.

결론 및 의의: INS-1 베타 세포에서 TNF-α의 전처리는 포도당 자극 인슐린 분비뿐만 아니라 그 외 분비 자극 물질에 대한 인슐린 분비도 현저하게 감소시켰다. INS-1 베타 세포에서 이러한 인슐린 분비 감소 현상의 원인으로는 포도당 자극 Ca\textsuperscript{2+}유입량의 감소 때문으로 생각되며 TNF-α에 의해 활성화된 NFkB의 신호 전달 체계가 포도당 자극 인슐린 분비 저하에 관련된 것으로 생각된다. 우리의 연구를 통해서 NFkB의 활성화가 제2형 당뇨병의 병인 중 하나인 인슐린 분비 저해를 유도할 수 있음을 보여주었다.

핵심어: Tumor necrosis factor-α (TNF-α), 포도당 자극 인슐린 분비 (GSIS), K\textsubscript{ATP} channel, L-type Ca\textsuperscript{2+} channel, NFkB, SN50
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I. IkBα 유전자 과발현 세포주

IV. 고 찰

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<tr>
<td>IDDM</td>
<td>Insulin Dependent Diabetes Mellitus</td>
</tr>
<tr>
<td>NIDDM</td>
<td>Non Insulin Dependent Diabetes Mellitus</td>
</tr>
<tr>
<td>GLUT2</td>
<td>Glucose Transporter 2</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-Protein Coupled Receptor</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>Inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>GSIS</td>
<td>Glucose stimulated insulin secretion</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor-κ Kappa B</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
</tr>
<tr>
<td>NIK</td>
<td>NFκB Inducing Kinase</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitory Kappa B</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-related kinase</td>
</tr>
<tr>
<td>Kir6.2</td>
<td>inward rectifier K channel subunits 6.2</td>
</tr>
<tr>
<td>SUR1</td>
<td>Sulfonylurea receptor 1</td>
</tr>
<tr>
<td>$K_{ATP}$ channel</td>
<td>ATP dependent K channel</td>
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Ⅰ. 서 론

A. 당뇨병의 정의 및 분류

우리의 인체는 음식물을 통해 에너지를 얻어 살아가고 그 에너지의 대부분은 포도당을 통해 만들어진다. 포도당이 체내에 흡수되면 혈중 포도당 (혈당)이 증가하고 증가된 혈당은 췌장에서 분비되는 호르몬인 인슐린에 의해 간, 골격근, 지방 조직으로 흡수되어 에너지로 사용된다. 에너지로 사용하고 남는 포도당은 인슐린에 의해 간에 흡수되어 글리코젠 (glycogen)이라 불리는 물질로 바뀌어 저장된다. 식사를 하지 않은 상태에서 세포가 에너지를 필요로 하게 되면 간에 저장되어 있던 글리코젠은 포도당으로 바뀌어 다시 혈관으로 내보내져서 다른 세포가 이용할 수 있게 된다. 이렇게 인슐린은 체내 혈당을 조절하여 대사의 항상성을 유지시키는데 중요한 역할을 한다.

그러므로 이러한 인슐린의 분비 및 작용이 저하되거나 글루카곤의 분비가 증가해서 혈중 포도당 농도가 기저농도에 비해 높은 상태로 지속적으로 유지되면 복합적인 병증을 나타내는데 그러한 상태를 당뇨병이라 한다. 당뇨병은 그 발병원인에 따라서 두 가지로 나뉘어진다. 첫째가 인슐린 의존형인 제1형 당뇨병 (Insulin Independent Diabetes Mellitus: IDDM)인데 이는 유전적 감수성 및 여러 환경인자가 작용하여 자가 면역반응을 일으켜 베타 세포가 파괴되어 인슐린 분비가 절대적으로 부족하게 되어 당뇨병에 걸린 경우이다. 둘째는 인슐린 비의존형인 제 2형 당뇨병 (Non Insulin Dependent Diabetes Mellitus : NIDDM)이다. 이는 인슐린 분비는 되지만 인슐린 저항성과 함께 상대적인 인슐린 분비 결핍으로 인해 혈당 조절이 안 되어서 당뇨병에 걸린 경우이다.
B. 제 2형 당뇨병


C. 인슐린 분비

1. 인슐린 생합성 및 분비

췌장 소도의 베타 세포는 체내의 대사상태에 따라 인슐린을 합성하고 분비하는 역할을 한다. 소도세포에는 췌타이드를 분비하는 내분비 세포들이 밀집되어 있는데 그 중 70%~90%를 차지하고 있는 것은 인슐린을 분비하는 베타 세포 (β cell)이다. 그 외 글루카곤을 분비하는 알파 세포 (α cell), 소마토스타틴을 분비하는 델타 세포 (δ cell), 췌장 폴리펩타이드를 분비하는 PP 세포 이렇게 4 가지 세포로 구성되어있다.

인슐린은 췌장의 베타 세포에서 생성 분비되는 췌타이드 호르몬으로서 포도당, 단백질, 지질등의 동화작용을 촉진하여 대사의 혼합성을 유지시키는 중요한 생리적 역할을 한다. 베타 세포에서 인슐린 생합성의 전반적인 과정을 살펴보면 포도당 저항에 의해서 인슐린 유전자에서 전연구인슐린 (preproinsulin)이 만들어지게
고 전구인슐린에서 24개의 아미노산이 잘려나가 전구인슐린 (proinsulin)이 되고, 전구인슐린에서 다시 4개의 아미노산이 잘려나가면서 51개의 아미노산을 가진 인슐린과 31개의 아미노산을 갖는 C-peptide로 변환된다.

2. 이상성과 박동성 인슐린 분비 양상

베타 세포에서 포도당에 의한 인슐린 분비는 1차 분비와 2차 분비로 나뉘어지는 이상성 분비 양상을 나타낸다 (Straub 등, 2002). 1차 분비는 베타 세포에 포도당 농도를 급격히 올렸을 때 인슐린 분비가 일시적으로 급격히 상승하는 시기를 말하며 대개 10분 내에 인슐린 분비가 기저치로 회복된다. 제 2차 분비는 포도당 농도를 서서히 증가시킬 때 포도당에 노출된 이후 25분에서 30분 이후부터 약 4시간까지 점진적으로 인슐린 분비가 이루어지는 시기이다. 이러한 현상은 in vitro에서 perifusion 실험을 통해서 알 수 있으며 in vivo의 경우 포도당의 정맥 주입을 통해서 알 수 있었다 (Curry 등, 1968). 이러한 이상성 인슐린 분비 양상은 인슐린낭의 구별된 pool의 존재와 포도당에 의해 활성화되는 신호들의 kinetics의 차이로 나타난다 (Henquin 등, 2003). 이렇게 합성된 인슐린은 Reserve pool, Docked pool, Readily releasable pool이라 불리는 인슐린 priming 과정을 통해서 분비된다 (Fig. 1) (Bratanova-Tochkova 등, 2002).

베타 세포에서의 포도당 자극 인슐린 분비의 또 다른 특징은 포도당 대사과정, 세포 내 Ca^{2+} 유입, 세포막 전위가 박동함 (oscillation)에 따라 인슐린 분비 형태에 박동성이 나타나는 것이다 (Porksen 등, 2002). 포도당에 대한 1, 2차 인슐린 분비의 감소와 박동성 인슐린 분비 형태에 이상이 생기는 것은 제 2형 당뇨병의 원인 중 하나이다.
Fig. 1. Granule pools in the $\beta$-cell.
3. 인슐린 분비 기작

(1) Triggering 경로

베타 세포에서 인슐린 분비를 위해서는 일차적으로 세포 내 Ca\(^{2+}\)이 증가하는 것이 필수적이다. (Fig. 2)의 Site 1에서 보듯이 포도당이 베타 세포에 있는 GLUT2 (glucose transporter 2)라고 하는 포도당 수송체에 의해 세포 내로 들어가면 해당작용과 TCA cycle을 통해서 ATP를 만들어낸다 (Site 2). 증가된 ATP/ADP 비율은 K\(_{\text{ATP}}\) channel을 닫음으로서 세포막의 탈분극을 일으킨다 (Cook 등, 1984). 이때 생긴 막전위 차에 의해 전압 의존적 Ca\(^{2+}\) channel이 열리게 되고 (Site 3) 세포외부의 Ca\(^{2+}\)이 대량으로 세포 내부로 유입되어 인슐린 분비를 유발시킨다 (Site 5) (Ashcroft 등, 1984; Wollheim, 1981).

K\(_{\text{ATP}}\) channel은 Kir 6.2 (inward rectifier potassium channel subunits 6.2) 와 SUR1 (Sulfonylurea receptor 1)과 같은 하위구조 단백질로 구성되어 있다. K\(_{\text{ATP}}\) channel을 닫음으로서 인슐린을 분비하는 물질로는 Glimepiride, Tolbutamide같은 Sulfonylurea류 제제와 Efaroxan, Phentolamine과 같은 Imidazoline 화합물들이 알려져 있는데 Sulfonylurea류 제제와 Imidazoline 화합물은 각각 K\(_{\text{ATP}}\) channel의 하위구조 중 하나인 SUR1과 Kir 6.2와 상호작용하여 인슐린 분비를 일으키는 것으로 밝혀져 있다 (Hoenig 등, 1986; Korytkowski 등, 2004; Ball 등, 2004; Efendic 등, 2002). 포도당 이외에도 고농도의 KCl은 세포 내로 유입되면서 세포막의 탈분극을 유발시켜서 인슐린 분비를 일으킬 수 있다 (Liu 등, 2003). 또한 세포 외부에 Arginine의 농도가 증가하면 cationic amino acid transporter (CAT2A)를 경유하여 베타 세포 내로 양이온이 유입되면서 탈분극을 유도하여 인슐린 분비를 유발시킬 수 있다 (Smith 등, 1997). 즉, 베타 세포 내에 Ca\(^{2+}\)을 급속히 증가시키는 경로가 1차 인슐린 분비를 일으키는 Triggering 경로이다.
(2) Amplifying 경로

포도당은 베타 세포에서 Triggering 신호와 함께 Ca^{2+}에 의한 인슐린 분비 효율을 증가시키는 Amplifying 신호를 만들어낸다. 이 경로는 주로 2차 분비에 관련된 신호 전달 경로 알려져 있다. 이 Amplifying 경로를 통한 인슐린의 지속적인 분비는 세포 내 Ca^{2+}의 증가를 유발시키는 Triggering 신호가 필수적이다 (Henquin 등, 2003).

Amplifying 신호를 활성화시키는 메개체는 아직까지 분명하게 밝혀져 있지 않은 않으나 몇 가지 대사 중간 산물이나 신호 전달 물질들로 생각하고 있다. 그 중 첫 번째는 malonyl co-A/long chain acyl-coA 가설로서 지속적인 포도당의 유입은 mitochondrial citrate의 양을 증가시키고 이것은 세포 내 citrate의 양을 증가시킨다. 그로 인해 malonyl co-A의 양이 증가하는데 이것이 CPT-1을 저해해서 지방산의 베타 산화를 감소시킨다. 결과적으로 세포 내 long chain acyl-coA가 양적으로 증가하여 인슐린 분비를 증가시킨다는 설이다 (Straub 등, 2002). 두번째 가설은 포도당에 의해 증가된 glutamate가 인슐린낭에 직접적으로 작용함으로써 인슐린 분비를 증가시킨다고 한다 (Corkey 등, 2000). 세 번째는 베타 세포 내의 ATP/ADP, GTP/GDP Level의 변화가 인슐린 분비를 조절하는 주요 요인이라고 주장하고 있다 (Bertrand 등, 2002). 그 외 Amplifying 신호로 PKC, PKA, Phospholipase A2, nitric oxide 생성, PI3Kinase 등이 알려져 있다 (Fig. 2의 Site4) (Detimary P 등, 1996).

Protein kinase C (PKC)의 활성에 따른 인슐린 분비는 Acetylcholine과 같은 호르몬에 의해 활성화 되는 신호 전달 경로가 대표적이다. G-protein coupled receptor가 호르몬에 의해 활성화 되면 phospholipase C가 활성화되어 Inositol 1,4,5-trisphosphate (IP_{3})와 Diacylglycerol (DAG)이 생성된다. 이때 생성된 IP_{3}는 소포체 막에 존재하는 IP_{3} 수용체 (Inositol 1,4,5-trisphosphate Receptor)와 작용하여 세포 내로 Ca^{2+}를 방출한다. 또 증가된 DAG는 PKC isoform을 활성화시켜서 인슐린을 분비한다 (Troitzka 등, 2002). DAG
analogue인 PMA (phorbol 12-myristate 13-acetate)는 PKC를 활성화시켜서 인슐린을 분비하는 것으로 알려져 있다 (Lee 등, 2003). Protein Kinase A (PKA)의 활성에 따른 인슐린 분비는 GLP-1이나 GIP같은 장관 내 호르몬에 의해 Adenyl cyclase가 활성화되는 신호 전달 경로이다. 장관 내 호르몬에 의해 GPCR이 활성화되면 Adenyl cyclase가 활성화되어 cyclic AMP (cAMP)가 다량 생산된다. 이 cAMP는 PKA와 cAMP-regulated guanine nucleotide exchange factors (Epac)과 같은 신호 전달 물질들을 활성화시켜 인슐린 분비를 촉진시킨다 (Troitza 등, 2002; Holz 등, 2004). Forskolin은 베타 세포에서 PKA를 활성화시켜 인슐린 분비를 일으키는 것으로 알려져 있다 (Lee 등, 2003).
Fig. 2. Schematic representation of insulin secretion pathways in β-cell.

site1: Glucose transport.

site2: Oxidative metabolism in β-cell

site3: Increase of β-cell [Ca^{2+}]i by blockade of K_{ATP} channels

site4: Stimulation of amplifying pathways in β-cell

site5: Exocytosis of insulin by Ca^{2+}
D. 제2형 당뇨병에서 인슐린 분비 이상

체적 베타 세포의 주요 기능은 체내 혈당의 향상성을 유지하기 위해서 인슐린을 합성하고 분비하는 것이다. 이러한 베타 세포가 지속적인 고혈당 상태에 노출되어있으면 포도당과 그 외 인슐린 분비 자극에 대한 1,2차 분비의 감소와 박동성 인슐린 분비 형태에 이상이 유발된다고 보고되어 있다 (Henquin 등, 2003). 베타 세포가 포도당 또는 그 외 인슐린 분비 자극에 지속적으로 노출되어 있으면 그 인슐린 분비 자극에 대한 베타 세포의 인슐린 분비 반응이 감소한다. 그리고 인슐린 분비 자극을 제거하게 되면 인슐린 분비 반응이 정상으로 회복되는 데 이러한 상태를 인슐린 분비의 Desensitization이라한다. 이때 인슐린 분비 반응의 감소가 세포 내 인슐린의 양적 감소를 동반하는 경우에는 베타 세포 Exhaustion이라 일컫는다 (Rustenbeck 등, 2004). 당뇨병이 오랜기간 지속되면 인슐린 분비 자극을 제거함에도 불구하고 인슐린 분비 반응이 더 이상 회복되지 않는 상태에 이르게 되고 이 상태는 베타 세포 기능이상과 구조의 변화를 동반한다. 즉, 베타 세포의 양적 감소로 이어져 되돌릴 수 없는 완전한 당뇨병으로 진행되는 것으로 알려져 있다 (Weir 등, 2001).

이러한 인슐린 분비 감소의 원인으로 밝혀져 있는 물질은 지속적인 고혈당과 증가된 FFA에 의해 생성된 ROS와 TNF-α, IL-1β 등의 cytokine이 있다 (Evans 등, 2003). 베타 세포가 지속적인 고혈당에 노출되면 세포 내 ROS, 단백질의 nonenzymatic glycation (Brownlee 등, 2000), 포도당 자가산화 등에 의해서 산화스트레스가 유발된다 (Wolff 등, 1991). 또한 베타 세포가 유리지방산 (free fatty acid : FFA)에 지속적으로 노출되어 있으면 mitochondrial uncoupling, 베타 산화가 증가하여 산화스트레스를 유발한다 (Wojtczak 등, 1993; Carlsson 등, 1999). 이와 더불어 고혈당과 유리지방산에 의한 산화스트레스는 스트레스 자극 신호 전달 경로를 활성화시켜 인슐린 분비를 저해한다. TNF-α, IL-1β는 활성화된 면역세포에서 분비되는 세포독성 cytokine으로서
당뇨병을 일으키는데 관여하고 있다는 사실이 알려져 있다 (Evans 등, 2003). 그 중 비만형 제 2형 당뇨병 환자에서 증가되어있는 adipokine 중의 하나인 TNF-α (Tumor necrosis factor-α)는 INS-1 베타 세포에서 포도당에 의한 인슐린 분비 (Glucose stimulated insulin secretion: GSIS)을 저해하는 것으로 밝혀져 있다 (Zhang S 등, 1995). 그러나 그 기작은 확실히 밝혀져 있지 않다.

E. TNF-α의 신호 전달 경로

TNF-α는 TNFR1과 TNFR2의 2개의 수용체와 결합한다. 그 중 TNFR1이 대표적인데 TNF-α와 TNFR가 결합하여 활성화되면 TNFR의 cytosolic domain인 death domain이 adaptor protein인 TRADD (TNFR associated DD protein)를 Recruiting한다. 그리고 TRADD는 TRAF-2와 FADD (FAS associated DD protein)를 recruit한다. 이 경로는 세포사와 관련되어있다 (Z.G. Liu 등, 1996). 반면 TRAF2는 NIK (NFκB Inducing kinase)를 recruiting하여 NFκB를 활성화 시키고 세포 생존에 관련하는 것으로 알려져 있다. 이 경로와는 별도로 TNF-α는 TRAF2를 통해 JNK와 SAPK를 활성화시키는 것으로 알려져 있다. TNF-α가 활성화 시키는 신호전달 물질에는 대표적으로 Nuclear factor Kappa B (NFκB), Jun N-terminal kinase (JNK), p38, Extracellular signal-related kinase (ERK)등이 알려져 있다 (Wulczyn 등, 1996). 이 중 Nuclear factor Kappa B (NFκB)는 innate & adaptive immunity, inflammation, stress response, B cell development, lymphoid organogenesis등 여러 방면의 유전자 발현에 관여하는 전사인자로 알려져 있다 (Hsu 등, 1996). 평소에 NFκB는 IκB에 의해 비활성화 상태로 존재하다가 NIK에 의해 활성화된 IKK (IκB kinase)가 IκB를 인산화시키고 인산화된 IκB는 NFκB에서 떨어져 나가 분해되고 NFκB는 활성화 상태로 바뀌어 핵 안으로 이동하여 전사인자로서 작용하게 된다 (Gutian 등, 2004). SN50은 NFκB
의 하위 구조인 p50의 nuclear localization sequence에 작용하는 Cell–permeable synthetic peptide로서 NFκB가 핵으로 이동하는 것을 특이적으로 저해하며 NFκB의 DNA 결합을 막는 것으로 알려져 있다 (Lin 등, 1995).

F. 연구목적

INS-1 베타 세포가 TNF-α에 노출되어 있을 때 GSIS가 감소되어 있었다. 그래서 본 연구는 TNF-α가 어떻게 베타 세포에서의 GSIS를 감소시키는지 그리고 기작을 밝히고자 한다. 베타 세포에서 TNF-α에 의해 GSIS가 저하되는 기작을 밝다면 인슐린 비의존형 당뇨병 (NIDDM)의 병인 중 하나인 인슐린 분비 결핍에 대해 이해하는데 도움이 될 것이다.
Fig. 3. TNF-α activated signaling pathway
Ⅱ. 재료 및 방법

A. 재료

1. 재료
세포 배양에 사용하는 Fetal bovine serum (GibcoBRL, Life Technology, NY)과 antibiotics antimycotics (GibcoBRL, Life Technology, NY)는 GibcoBRL, Life Technology에서 구입하였다. 세포 배양에 사용하는 RPMI 1640은 sigma(sigma, St. Lois)에서 구입하였다. 체장 소도세포 분리에 사용한 Collagenase P는 Roche(Roche diagnostics GmbH, Mannheim, Germany)에서 구입하였고, Ficoll은 sigma에서 구입하였다. Recombinant Murine TNF-α는 R&D systems에서 구입하였다. Dentrolene, Xestospongion C (XeC), Nifedipin, Diazoxide, KN93, EGTA/AM, BAPTA/AM, H89, GF109203X, GO6976, GO6983, PD98059, SB203580, SP600125, SN50, NMMA등의 저해제들은 Calbiochem (Calbiochem, Germany)에서 구입하였다. L-leucine, Glutamate, pyruvate, Glimipiride, Tolbutamide, Phentolamine, Efaroxan등은 sigma에서 구입하였다. Western blot에 사용된 Kir 6.2와 SUR 1의 항체는 Santa Cruz (Santa Cruz Biotechnology, CA)에서 구입하였고 α1D의 항체는 (Calbiochem, Germany)에서, 그리고 IκBα 항체는 Cell signaling (Cell signaling Technology, USA)에서 구입하였다. 세포 내 ATP측정에 사용되었던 Cell Titer-Glo Luminescent Cell Viability Assay Kit는 Promega에서 구입하였다. 인슐린 측정에 사용되었던 Rat insulin assay Kit (Linco Research, St. Louis, MO)는 Linco에서 구입하였다.
<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dantrolene</td>
<td>RyR inhibitor</td>
</tr>
<tr>
<td>Diazoxide (DZX)</td>
<td>$K_{ATP}$ channel opener</td>
</tr>
<tr>
<td>H89</td>
<td>PKA inhibitor</td>
</tr>
<tr>
<td>GF109203X</td>
<td>Pan PKC inhibitor</td>
</tr>
<tr>
<td>GO6976</td>
<td>cPKC inhibitor</td>
</tr>
<tr>
<td>GO6983</td>
<td>cPKC, nPKC inhibitor</td>
</tr>
<tr>
<td>PD98059</td>
<td>ERK inhibitor</td>
</tr>
<tr>
<td>SB203580</td>
<td>p38 inhibitor</td>
</tr>
<tr>
<td>SP600125</td>
<td>JNK inhibitor</td>
</tr>
<tr>
<td>SN50</td>
<td>NFkB inhibitor</td>
</tr>
<tr>
<td>NMMA</td>
<td>iNOS inhibitor</td>
</tr>
<tr>
<td>Bay K8644</td>
<td>L-type $Ca^{2+}$ channel agonist</td>
</tr>
<tr>
<td>KN93</td>
<td>CaM kinase II inhibitor</td>
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<tr>
<td>Xestospongion C</td>
<td>IP$_3$ inhibitor</td>
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<tr>
<td>Nifedipine</td>
<td>L-type $Ca^{2+}$ channel blocker</td>
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<tr>
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<tr>
<td>NAC</td>
<td>anti oxidant</td>
</tr>
</tbody>
</table>
B. 방법

1. 세포주 및 세포 배양

INS-1 세포주와 췌장 소도 세포는 11mM 포도당이 포함된 RPMI 1640 배지에 10% Fetal bovine serum과 100IU/ml penicillin과 100μg/ml streptomycin의 항생제를 첨가하여 5% CO2와 37℃의 온도를 유지하면서 배양하였다.

2. 췌장 소도 세포 (Pancreas islet) 분리

췌장 소도 세포는 collagenase digestion 방법으로 분리하였다. 8~10주 된 웅성 Sprague-Dawley 쥐 (300g)의 common bile duct에 0.75mg/ml의 collagenase 를 10ml 주사하고 췌장을 분리하였다. 37℃ water bath에서 약 10분 정도 두면서 digestion 시킨 후, 차가운 HBSS를 20ml 넣어 효소의 작용을 중지 시키고 나서 pipetting을 세차계 20회 정도 해 준 후, 600㎛ mesh에 걸러서 digestion되지 않은 exocrine 세포들을 제거하였다. 걸러진 용액은 50g로 원심 분리하여 상등액을 버리고 절라낸 HBSS를 넣고 섞어 50g로 원심 분리하여 씻어내는 과정을 3회 반복하였다. 남은 절라낸은 25% 용액에 섞은 후, 그 위로 23%, 21.5%, 20.5%, 11%의 ficoll층을 만들고 3500rpm에서 10분간 원심 분리하였다. 11%와 20.5% 사이 층에 걸린 췌장 소도 세포를 분리하여 RPMI 1640로 세척한 후, RPMI 1640 (10% fetal bovine serum과 1%항생제 포함) 배지로 약 12시간 배양하였다.

3. MTT 측정

INS-1 베타 세포를 96Well plate에 well당 5×10^4로 분주하고 24시간 동안 배양한 후, TNF-α를 농도별로 24시간 처리하였다. MTT (3-4,5-dimethythiazol-2-yl) −2,5 −(diphenylte-trazoillum)를
0.5mg/ml 농도로 3시간동안 37℃에서 반응시킨 후, 상등액을 버리고 isopropanol로 2분 동안 녹였다. Microplate Reader Benchmarker (Bio Rad, Japan)로 흡광을 측정하였다.

4. 인슐린 정량

INS-1 세포는 RPMI 1640 (10% fetal bovine serum과 1%항생제 포함) 배지로 24well dish에 1.5×10^5개를 깔고 24시간 배양하였다. TNF-α와 각각의 저해제들을 함께 24시간 처리한 후에 인슐린 분비량을 조사하였다. 0.2mM 포도당이 첨가된 KRB buffer (NaHCO_3 24mM, MgCl_2 1.2mM, Hepes 1mM, NaCl 129mM, KCl 4.8mM, KH_2PO_4 1.2mM, CaCl_2 2.5mM, 0.2% BSA)로 1시간 동안 전배양을 수행한 후, 먼저 TNF-α와 저해제들이 포함된 준비해 두었던 배지를 1ml씩 각 well에 넣어주었다. 2시간 동안의 배양이 끝난 뒤, 각상등액을 200㎕씩 모아 Rat insulin assay kit로 Radioimmunoassay 방법을 통해 인슐린을 측정하였다. 세포 내 존재하는 인슐린량을 측정하기 위해서 acid-ethanol (ethanol/H_2O/1M의HCl, 790:10:100)을 각 well당 200㎕씩 넣고 4℃에서 12시간 동안 두었다. 그 추출물을 모아서 700g에서 3분간 원심분리 후 Rat insulin assay Kit를 사용하여 Radioimmunoassay법으로 인슐린을 측정하였다.

5. RT-PCR

INS-1 세포를 RPMI 1640 (10% fetal bovine serum과 1%항생제 포함) 배지로 12well plate에 3.1×10^5개씩 분주해서 37℃, 5% CO_2에서 20시간 동안 배양한 후, 각각의 약제들을 처리하였다. 18~24시간 이후에 세포를 PBS로 2회 씻어준 후, 각각의 약제들을 처리하였다. 18~24시간 이후에 세포를 PBS로 2회 씻어준 후, RNA Zol-B를 이용해 전체 RNA를 뽑고 정량하였다. 1㎍의 RNA를 1000U AMV 0.5㎕, 2.5mM dNTP 4㎕, Random 9mer 1㎕, RNase inhibitor 0.5㎕, MgCl_2 4㎕, 10배 RT buffer를 이용해서 30℃ 10분, 42℃ 30
분, 99℃ 5분 동안 역전사 반응을 시킨다. 이렇게 얻어진 cDNA로 PCR을 수행하였다. 사용한 primer는
Cyclophilin (Foward) : 5'-CCA AAg ACA gCA gAA AAC TT3'
Cyclophilin (Reverse) : 5'-gAA ATT AgA gTT gTC CAC Ag3'
iNOS (Foward) : 5'-gCA gAA TgT gAC CAT CAT gg3'
iNOS (Reverse) : 5'-ACA ACC TTg gTg TTg AAg gC3'
GLUT2 (Foward) : 5'-Tgg gTT CCT TCC AgT TCg-3'
GLUT2 (Reverse) : 5'-Agg CgT CTg gTg TCg TAT g-3'
Glucokinase (Foward) : 5'-TgA Cag AgC CAg gAT ggA g-3'
Glucokinase (Reverse) : 5'-TCT TCA CgC TCC ACT gCC-3'
Kir6.2 (Foward) : 5'-CCA TgT CCT TCC TgT gCA gCT g3'
Kir6.2 (Reverse) : 5'-CCg CAA CTC Agg ACA Agg AAT C-3'
Sur1 (Foward) : 5'-TTg CTg AAA CTg Tgg AAg gAC TCA C-3'
Sur1 (Reverse) : 5'-TTC Agg ACC ATC ACT Agg TCT gCA C-3'
α1D (Foward) : 5'-gAg gCA AAC TAT gCA AgA ggC AC-3'
α1D (Reverse) : 5'-CTg ACT CAg ATA TAg TAg CTg AAg-3'
β3 (Foward) : 5'-CCA TCC CTg gAC TTC Ag-3'
β3 (Reverse) : 5'-CTA ACg CCA ATC TAA CTC C-3' 이다.

이제 얻은 PCR 반응물을 0.8% agarose gel에서 전기영동하여 EtBr로 염색하여 사진을 찍었다.

6. 세포 내 Ca^{2+}량 측정
세포 내 Ca^{2+} 측정은 fura 2와 dual excitation wavelength fluorescence microscopy를 이용했다. INS-1세포를 staining buffer에 1μM Fura -2AM (sigma, St Lois), Me2SO 0.09g/l, Pluronic F-127 0.018g/l (molecular Probe, Eugene, OR)로 30분 동안 염색한 후 세포를 Nicon Diaphot-300

7. 세포 내 ATP량 측정

INS-1 세포를 24well plate에 2×10^5으로 분주한 후, 20시간동안 배양했다. 비교군에 TNF-α를 농도별로 24시간 동안 처리했다. 그 후 포도당이 없는 KRB buffer로 세포를 3회 씻어준 후, 0.2mM 포도당이 포함된 KRB buffer로 1시간 동안 전배양을 했다. 각각 0.2mM, 16.7mM 포도당이 들어있는 KRB buffer를 well당 1ml씩 처리한 후, 적정 시간 후 상등액을 모두 거둬들이고 ATP합성관련 효소 활성을 억제하기 위해서 5% trichloroacetic acid in PBS를 0.5ml 참가했다. 세포들을 pipetting하여 모으고 5분간 300g로 원심분리하여 상등액을 버리고 침전된 세포에 5% trichloroacetic acid in PBS 0.2ml을 넣고 sonication한 후에 얼음에 두었다. 10분간 13000rpm으로 원심분리하고 0.1ml의 상등액을 Ependorf tube에 옮긴 후, trichloroacetic acid를 제거하기 위해서 1ml의 diethyl ether를 섞은 후 상등액을 제거하였다. 위 과정을 3회 시행하여 trichloroacetic acid를 완전히 제거하였다. 0.1ml의 sample을 buffer A (20mM HEPES, 3mM MgCl2, KOH로 PH7.75) 에 희석하여 Cell Titer-Glo Luminescent Cell Viability Assay Kit (Promega)을 사용하여 ATP량을 측정하였다.

8. Western blotting

INS-1 세포를 6 well plate에 well당 1×10^6개씩 분주하고 20시간 동안 배양하였다. TNF-α 와 약제들을 24시간 처리한 후 각각의 세포들을 수거하여 RIPA buffer (1% triton X-100, 1% sodium deoxycholate, 50mM NaCl2,
50mM tris-HCl, 1mM sodium vanadate, 2mM PMSF)를 이용하여 세포로부터 단백질을 분리, 정량하여 sample buffer (187mM tris-HCl pH6.8, 10% SDS, 30% glycerol, 100mM DTT, 0.3% bromophenol blue)에 희석하여 5분간 끓인 다음 8% SDS-polyacrylamid gel에서 전기영동 하였다. Gel에서 NC membrane으로 이동시키고 5% skim milk로 blocking하였다. 각각의 항체와 반응시키고 horseradish peroxidase linked 2차 항체로 반응시켜서 ECL system으로 조사하였다.

9. 세포막 단백질 분리

INS-1 세포를 60mm dish plate에 well당 1.5×10⁶개씩 분주하고 20시간 동안 배양하였다. TNF-α는 농도별로 24시간 처리하였다. 각각의 세포들은 수거하여 Lysis buffer (1% triton X-100, 1% sodium deoxycholate, 50mM NaCl₂, 50mM tris-HCl, 1mM sodium vanadate, 2mM PMSF, protease inhibitor cocktail)를 이용하여 세포로부터 단백질을 분리한다. Sonication (10%로 5초씩 5번: bubble이 생기지 않도록 주의) 후, 얼음에 5분간 둔다. 700g로 원심분리 하여 첨전물(핵, 분해되지 않은 세포등)은 버리고 상등액을 거둬 8000g로 원심분리 한 후에 첨전물(세포막 단백질)을 정량하여 2X sample buffer (187mM tris-HCl(pH6.8), 10% SDS, 30% glycerol, 100mM DTT, 0.3% bromophenol blue)에 희석하여 5분간 끓인 다음 8% SDS-polyacrylamid gel에서 전기영동 한다. 남은 상등액은 단백질을 첨전시킨 후, 정량하여 조사하였다.

10. Transfection

INS-1 세포를 6well plate에 well당 8×10⁶개씩 분주하고 20시간 동안 배양하였다. Eppendorf tube에 serum 없는 RPMI 600ul를 넣고 원하는 DNA
4.5㎍을 섞어준 다음 9ul의 lipofectamine을 더해 pipet으로 부드럽게 섞어준 후, 30분 정도 상온에 두었다. Transfection 시킬 세포를 serum 없는 RPMI로 2회 씻어준 다음, serum free RPMI를 well당 1.4ml씩 분주하고 준비해둔 DNA와 lipofectamine이 혼합된 배지를 세포위로 천천히 뿌려주었다. 4~6시간 배양시키고 나서 RPMI 1640 (10% FBS 포함)으로 교체하였다. 48~72시간 내에 세포를 100mm culture dish로 옮겨서 24시간 배양한 후, 배지에 63.8mg/ml 의 G418 을 넣어서 48시간 배양하였다. 그 이후에 살아남은 세포들을 배양하여 stable clone 세포를 얻었다.
Ⅲ. 결과

A. INS-1 베타 세포에서 TNF-α가 GSIS에 미치는 영향

INS-1 베타 세포에서 TNF-α가 GSIS를 감소시킨다는 사실은 이미 밝혀져 있다 (Zhang 등, 1995). 우리 실험체제에서도 TNF-α에 의한 GSIS의 감소 양상을 확인해보고자 농도별, 시간별 인슐린 분비 양상을 조사해 보았다. (Fig. 4A)에서 보는 바와 같이 TNF-α를 1.25ng/ml, 2.5ng/ml, 5ng/ml, 10ng/ml, 20ng/ml 농도로 24시간 동안 처리한 후, 2시간 동안의 GSIS를 조사했다. 1.25ng/ml의 TNF-α 농도부터 그 농도가 증가함에 따라 GSIS가 감소됨을 확인했다. TNF-α 농도를 10ng/ml을 처리하였을 때는 GSIS가 50%이상 감소되어 있었고 그 이상의 농도에서는 동일하게 GSIS가 감소됨을 확인했다 (Fig. 4A). (Fig. 4B)에서는 TNF-α에 의해 GSIS가 감소되는 시간을 알아보기 위해서 시간별로 10ng/ml의 농도의 TNF-α를 처리해보았다. 그 결과, TNF-α를 처리하고 1시간이 흐르면 GSIS가 시간에 비례하여 감소되었고 TNF-α를 처리한 12시간 이후부터는 GSIS가 50%이상 감소되어있는 것을 확인하였다. 24시간 이후부터는 12시간에의 감소 양상과 동일한 결과를 얻었다. GSIS를 수행하는 2시간 동안에 인슐린 분비양상을 조사하기 위해서 0분, 30분, 60분, 120분에 각각 인슐린을 얻어서 시간별 분비량을 조사해 보았다. TNF-α를 처리한 INS-1 세포에서의 GSIS는 대조군에 비해 분비량이 총 50%이상 감소되어 있었다 (Fig. 4C). 그리고 이러한 GSIS의 감소 현상은 TNF-α를 제거한 뒤 6시간 후에 회복되었다 (Fig. 4D).

TNF-α는 IL-1β (Interleukin-1β), IFN-γ (Interferon-γ)와 같은 cytokine과 같이 베타 세포에 독성을 나타내는 것으로 알려져 있다 (Mandrup-Poulsen T, 2003) 따라서 우리가 처리한 20ng/ml의 TNF-α의
세포독성을 MTT 분석을 통한 세포 활성도를 조사해 보았더니 TNF-α는 단독으로 INS-1 베타 세포에 독성을 나타내지 않는 것으로 나타났다 (Fig. 5).
Insulin (fold)

A.

Control

B.

C.

D.

Insulin (ng/ml)
Fig. 4. Pattern of TNF−α−induced GSIS inhibition in INS−1 cell

A: After treatment of TNF−α (1.25, 2.5, 5, 10, 20 ng/ml)) for 24h, INS−1 cells were preincubated with KRB buffer containing 0.2mM glucose for 1h. Insulin concentration were measured 2h after 16.7mM glucose treatment. B: After treatment of TNF−α (10ng/ml) at distinct time, Which was performed in the same experiment. C: After treatment of TNF−α (10ng/ml) for 24h, INS−1 cells preincubated with KRB buffer containing 0.2mM glucose. Time course of insulin release were measured 2h after 16.7mM glucose treatment. D: After treatment of TNF−α (10ng/ml) for 24h, medium containing TNF−α were removed. INS−1 cells were washed and incubated with RPMI 1640. Data are representative of five independent experiments and are expressed as mean ± SD.
Fig. 5. Effect of TNF-$\alpha$ on INS-1 cell viability. After TNF-$\alpha$ (1.25, 5, 10, 20 ng/ml) treatment on INS-1 cell for 36h. Cell viability was determined by MTT assay. Data are representative of three independent experiments and are expressed as mean ± SD.
B. TNF-α가 인슐린 분비 자극 물질에 의한 인슐린 분비에 미치는 영향

TNF-α가 포도당 이외에 인슐린 분비 자극 물질들에 의한 인슐린 분비에 어떤 영향을 미칠 것인지를 알아보고자 이 실험을 수행하였다. INS-1 베타 세포를 10ng/ml의 TNF-α로 24시간 동안 처리하였다. 베타 세포 포도당 대사 과정 중에 바로 TCA 회로에 에너지 합성 대사물질로 사용되는 것으로 알려져 있는 L-leucine과 Glutamate를 각각 또는 동시에 같이 처리해서 2시간 동안의 인슐린 분비를 조사했다. (Fig. 6)에서 결과와 같이 L-leucine단독에 의한 인슐린 분비는 TNF-α에 의해 저해 받았으며 Glutamate 단독에 의해서는 인슐린 분비가 되지 않았다. 이 두 물질을 동시에 처리해서 인슐린 분비를 조사해보았더니 포도당에 의한 인슐린 분비 양상과 동일하게 감소하는 것으로 나타났다. 그리고 TNF-α에 의한 인슐린 분비가 50% 이상 감소되었다. 포도당 대사과정 중의 중간산물인 Pyruvate의 경우에도 인슐린분비가 TNF-α에 의해 20~30%까지 감소됨을 확인하였다 (Fig. 6). 포도당 대사과정에 의한 ATP 생성과정을 거치지 않고 바로 K<sub>ATP</sub> channel을 닫음으로서 인슐린 분비를 자극하는 Sulfonylurea류의 일종인 Glimepiride와 Tolbutamide도 TNF-α에 의해 인슐린 분비가 저해되었다. Imidazoline계열인 Phentolamine과 Efaroxan에 의한 인슐린 분비도 약 50%정도 저해되었다 (Fig. 7). L-type Ca<sup>2+</sup> channel에 직접 작용하여 세포 내 Ca<sup>2+</sup>유입을 증가시킴으로써 인슐린 분비를 유발시키는 Bay K8644에 의한 인슐린 분비 또한 감소하는 것으로 나타났다. 반면 (Fig. 8)에서 보는 것처럼 고농도의 KCl에 의해서 인슐린 분비를 유발 시켰을 경우에는 인슐린 분비는 TNF-α 전처리에 의해 인슐린 분비 저해 현상이 약간 나타났다. 이 KCl이 고농도에서 세포입장에서는 과탈분극상태로 인슐린 분비를 유발할 수도 있으므로 KCl이 탈분극을 통해 인슐린 분비를 일으킬 수 있는 더 낮은 KCl 농도하에서 TNF-α에 의한 인슐린 분비 저해 효과를 조사해 보았다. 그 결과 (Fig. 8)에서
15mM의 KCl에 의한 인슐린 분비는 TNF-α에 의해 저해 받는 것을 확인하였다. 그리고 (Fig. 9)에서 PKC, PKA의 활성화에 의해서 인슐린 분비를 유발 시켰을 경우에는 TNF-α의 전처리는 인슐린 분비를 저해시키지 않았다.
Fig. 6. Effect of TNF–α on ATP generating agent – stimulated insulin secretion  INS–1 cells were cultured for 24h in TNF–α (10ng/ml) containing medium. Following 1h preincubation with buffer containing 0.2mM glucose (LG), effect of 16.7mM glucose (HG), 20mM L–Leucine (Leu), 10mM glutamate (Gln), 20mM sodium pyruvate (Pyr) on insulin secretion were investigated after 2h incubation.
Fig. 7. Effect of TNF-\(\alpha\) on \(K_{ATP}\) channel closing agent–stimulated insulin secretion INS-1 cells were cultured for 24h in TNF-\(\alpha\) (10ng/ml) containing medium. Following 1h preincubation with buffer containing 0.2mM glucose (LG), effect of 16.7mM glucose (HG), 300 \(\mu\)M Tolbutamide (Tol), 3 \(\mu\)M Glimepiride (Gli), 200 \(\mu\)M Efaroxan (Efa), 50 \(\mu\)M Phentolamine (Phen) on insulin secretion were investigated after 2h incubation.
Fig. 8. Effect of TNF-α on Ca^{2+} releasing agent–stimulated insulin secretion. INS−1 cells were cultured for 24h in TNF−α (10ng/ml) containing medium. Following 1h preincubation with buffer containing 0.2mM glucose (LG), effect of 16.7mM glucose (HG), 15mM KCl, 10 μM Bay K8644 on insulin secretion were investigated after 2h incubation.
Fig. 9. Effect of TNF-α on PKC, PKA activating agent-stimulated insulin secretion. INS-1 cells were cultured for 24 h in TNF-α (10 ng/ml) containing medium. Following 1 h preincubation with buffer containing 0.2 mM glucose (LG), effect of 16.7 mM glucose (HG), 500 nM PMA, 20 μM Forskolin on insulin secretion were investigated after 2 h incubation.
C. TNF-α에 의한 인슐린 생합성량 변화

INS-1 베타 세포가 TNF-α 전처리에 의해 인슐린 분비가 현저하게 감소되는 것으로 나타났는데 감소되는 기작을 알고자 먼저 인슐린 생합성량의 변화를 mRNA와 단백질 수준에서 조사해 보았다. (Fig. 10A)에서와 같이 TNF-α를 5ng/ml, 10ng/ml, 20ng/ml의 농도로 24시간 동안 처리한 후 인슐린 유전자 발현량을 RT-PCR 방법으로 조사해 보았다. 그리고 TNF-α가 세포에 농도별로 정확히 작용했는지 알아보기 위해서 iNOS 유전자를 대조군으로 사용하였다. 그 결과 iNOS 유전자 발현량은 TNF-α의 농도에 따라 현저하게 증가한 반면 인슐린 유전자 발현량은 변화가 없었다. (Fig. 10B)에서 세포 내 존재하는 인슐린의 양에 변화가 있는지 조사해 보기 위해서 시간에 따른 세포 내 인슐린량을 측정하여 보았다. 각각 0, 1, 6, 12, 24, 36 시간에 따라 10ng/ml의 TNF-α를 처리한 후, 세포 내 인슐린량을 측정하였다.

이 실험결과 TNF-α는 세포 내 인슐린량에는 영향을 끼치지 않는 것으로 나타났다. 이에 따라 INS-1 베타세포에서 TNF-α에 의한 GSIS의 감소는 인슐린 생합성량의 변화와는 관계가 없음을 확인하였다.

D. TNF-α가 베타 세포 당대사에 미치는 영향

포도당에 의한 인슐린 분비는 포도당 대사를 통한 INS-1 베타세포에서 포도당 대사의 변화가 TNF-α에 의한 GSIS의 저해에 관련되어있는지 알아보기 위해서 포도당을 세포 내로 유입시키는 포도당 수송체인 GLUT2와 대표적인 당대사 조절 물질인 Glucokinase의 유전자 발현량을 조사해 보았다. TNF-α를 5ng/ml, 10ng/ml, 20ng/ml 농도로 INS-1 세포에 24시간 동안 처리한 후, 각각의 유전자 발현량을 조사하였다. 그 결과 TNF-α가 고농도일 때도 Glucokinase와 GLUT2의 유전자 발현량에는 변화가 없는 것으로 나타났다.
(Fig. 11). 베타 세포에 유입된 포도당이 해당과정을 거쳐 만들어내는 ATP/ADP 비율의 변화는 인슐린 분비의 조절에 중요하므로 TNF-α가 ATP의 합성에 영향을 미치는지 알아보고자 세포 내 ATP량을 측정하여 보았으나 (Fig. 12)에서 보는 바와 같이 변화가 없었다. 결과적으로 INS-1 세포에서 포도당 유입으로부터 해당과정을 거쳐 ATP를 생성하는 당대사 경로의 이상과 TNF-α에 의한 GSIS의 감소와는 관련이 없음을 확인하였다.
Fig. 10. Effect of TNF-α treatment on insulin expression  A: After TNF-α (5, 10, 20 ng/ml) treatment on INS-1 cell for 24h, total RNA was isolation and subjected to RT-PCR with the primers specific for cyclophilin, iNOS, insulin. The PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. B: After TNF-α (10ng/ml) treatment on INS-1 cell for 24h, The cellular insulin content was extracted by incubation in 1ml acid-ethanol. Assays were performed by I\textsuperscript{125} radioimmunoassay method. Data are representatives of three independent experiments and are expressed as mean ± SD.
Fig. 11. Effect of TNF−α on glucose metabolism related molecules

After TNF−α (5, 10, 20 ng/ml) treatment on INS−1 cell for 24h, total RNA was isolation and subjected to RT−PCR with the primers specific for cyclophilin, glucokinase, GLUT2. The PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. Data are representative of five independent experiments and are expressed as mean ± SD.
Fig. 12. Effect of TNF-α on ATP production in INS-1 cell.

A: ATP level were measured by Luminescent assay method dose dependently. B: INS-1 cell treated with and without TNF-α (10ng/ml) incubated for 24h in RPMI 1640 medium. INS-1 cells were treated with or without glucose containing KRB buffer. After incubation for 5min, the ATP concentration of the cellular extracts
were measured by Luminescent assay method.

E. TNF-α가 포도당 자극 Ca^{2+} 유입량에 미치는 영향

INS-1 베타 세포가 포도당 자극을 받아 인슐린을 분비하기까지에는 세포 내 Ca^{2+} 양의 조절이 매우 중요하다. 그래서 TNF-α가 포도당 자극 Ca^{2+}유입량에 어떤 영향을 미치는지 알아보기 위해서 Delta Scan dual monochromator system (Photon Technology International, Monmouth Junction, NJ)을 이용하여 세포 내 Ca^{2+}유입량을 측정하였다.

(Fig. 13A)는 INS-1 베타 세포에서 TNF-α가 단독으로 어떤 영향을 주는지 알아보고자 실험해보았다. 그 결과 TNF-α가 단독으로 세포 내 Ca^{2+}유입량을 증가시키는 것을 확인하였다. (Fig. 13B-up)는 대조군으로서 INS-1 베타 세포에 0.2mM 포도당을 포함한 배지에서 16.7mM의 포도당을 포함한 배지로 교체하여 포도당 자극 Ca^{2+}유입량을 측정한 것이다. 세포 내 Ca^{2+}농도가 5분여 만에 기저치에 비해서 약 2.5배 이상 증가함을 알 수 있다. 반면 (Fig. 13B-down)에서 보듯이 10ng/ml의 TNF-α를 24시간 처리한 경우 세포 내 포도당 자극 Ca^{2+}유입량 증가율이 대조군에 비해 현저하게 감소하였음을 확인하였다. 여기서 특이한 점은 TNF-α에 의해 전체적인 세포 내 포도당 자극 Ca^{2+}유입량도 감소하였지만 그에 따른 포도당 자극 Ca^{2+}유입의 박동성도 감소하였다. 이 결과로 TNF-α에 의한 인슐린 분비 저해 기작은 포도당 자극 Ca^{2+}유입량의 감소와 관련이 있음을 알 수 있었다.
A.

B.
Fig. 13. Effect of TNF-α on intracellular Ca²⁺ influx induced by glucose.

A: Intracellular Ca²⁺ changes were assessed by Fura-2 fluorescence measurements in cells maintained in normal Ca²⁺-containing medium. INS-1 cells stained with Fura-2AM of 50μM for 1h. The data represent intracellular Ca²⁺ change with time course. show the point for the addition of 10ng/ml TNF-α. B(up): The data represent intracellular Ca²⁺ change with time course. show the point for the addition of 16.7mM glucose. The red arrow show the point for the addition of 16.7mM glucose. B(down): After TNF-α (10ng/ml) treatment on INS-1 cell for 24h, Intracellular Ca²⁺ changes were measured by equal method. Data are representative of three independent experiments and are expressed as mean ± SD.
F. TNF-α에 의한 Ca^{2+} 유입량의 감소 작용점 조사

앞서 실험에서 보았듯이, TNF-α에 의한 GSIS의 감소 기작은 세포 내 포도당 자극 Ca^{2+}의 유입량의 감소와 관련이 있다. 베타 세포의 세포 내 Ca^{2+} 농도 증가시키는 경로는 Ca^{2+}이 세포 외부에서 유입되는 경로와 세포 내에 존재하는 소포체나 미토콘드리아와 같은 세포 내 소기관에서 세포내부로 유입되는 경로로 나누어진다. 그래서 증가된 기저 Ca^{2+}농도가 어디로부터 유입된 것인지 알아보기 위해서 각각의 저해제를 사용하여 실험하였다.

5μM 의 Dantrolene (RyR inhibitor)과 20μM의 Xestospongic C (IP_{3}R inhibitor), 300μM 의 Diazoxide (K_{ATP} channel opener), 10μM의 Nifedipine (L-type Ca^{2+} channel blocker) 을 각각 10ng/ml의 TNF-α와 함께 24시간 처리한 후, GSIS를 조사하였다. Diazoxide와 Nifedipine 을 처리한 세포는 TNF-α에 의해 감소되었던 인슐린 분비가 100% 회복됨을 확인했다 (Fig.14). 이 결과로 TNF-α에 의한 Ca^{2+}의 유입량의 감소는 K_{ATP} channel이 닫힘으로써 그에 따른 세포막의 탈분극에 의해 열리게 되는 L-type Ca^{2+} channel을 통한 기저 Ca^{2+}농도의 증가 때문임을 확인하였다.
Fig. 14. Diazoxide and Nifedipine protect against impaired insulin secretion elicited by TNF-α. INS-1 cells were cultured for 24h at
11mM RPMI medium with or without TNF-α (10ng/ml), 5μM Dantrolene (Dant), 20μM Xestospongin C (XeC), 300μM Diazoxide (DZX), 10μM Nifedipine (Nif). Following 1h preincubation with buffer containing 0.2mM glucose, GSIS were investigated after 2h incubation.

G. TNF-α가 Channel의 양적 변화에 미치는 영향

INS-1 베타 세포에서 전압 의존적인 L-type Ca\(^{2+}\) channel을 통한 외부 Ca\(^{2+}\) 유입량의 감소가 TNF-α에 의한 GSIS의 감소에 중요한 현상으로 확인되었으므로 외부 Ca\(^{2+}\) 유입에 관련된 Channel의 양적인 변화를 조사했다. TNF-α를 0, 2.5, 5, 10ng/ml의 농도로 24시간 동안 처리하고 K\(_{\text{ATP}}\) channel을 구성하고 있는 하위 구조인 Kir 6.2, SUR 1과 전압 의존적인 L-type Ca\(^{2+}\) channel을 구성하고 있는 α1D, β3의 유전자 발현량을 조사하였다. (Fig.15)에서 보는 바와 같이 이들 channel 유전자 발현량에는 변화가 없는 것으로 나타났다. 그리고 이들의 총단백질량은 Western blotting 방법을 통해서 조사하였다 (Fig.16). TNF-α를 0, 2.5, 5, 10ng/ml의 농도로 처리하고 총 K\(_{\text{ATP}}\) channel 단백질의 양을 조사하여 보았으나 변화가 없었다. 결과적으로 베타 세포에서 TNF-α에 의한 전압 의존적인 L-type Ca\(^{2+}\) channel을 통한 외부 Ca\(^{2+}\) 유입량의 감소는 channel의 양적인 변화와는 관련이 없는 것으로 확인되었다.
Fig. 15. Effect of TNF–α on gene expression of K<sub>ATP</sub> channel and L-type Ca<sup>2+</sup> channel After treatment of (2.5, 5, 10 ng/ml) TNF–α in INS–1 cell for 24h, total RNA was isolation and subjected to RT–PCR with the primers specific for cyclophilin, Kir 6.2, SUR 1, α1D, β3. The
PCR products were separated on a 1.2% agarose gel and visualized by ethidium bromide staining.

Fig. 16. Effect of TNF-α on protein expression of K\textsubscript{ATP} channel and L-type Ca\textsuperscript{2+} channel. INS-1 cells were incubated in TNF-α of distinct concentration for 24h. Cell lysates were made, and equivalent amounts of cellular proteins were separated by 10% SDS-PAGE, blotted, and then
probed with the Kir 6.2, SUR 1, α1D antibody.

H. TNF-α에 의한 인슐린 분비저해에 관련된 신호전달 물질 조사

베타 세포에서 인슐린이 분비되기까지에는 많은 신호전달 물질이 존재한다. TNF-α가 활성화 시키는 신호 전달 물질과 TNF-α가 Ca\(^{2+}\) channel 활성도를 조절하는 신호 전달 물질에 미치는 영향이 GSIS 감소 기작에 관련되어있는 지 확인해 보고자 저해제 실험을 수행하였다. 기존 논문에 의하면 심장 세포에서 PKA나 PKC가 Ca\(^{2+}\) Channel이 열리고 닫히는 것을 조절하는 것으로 밝혀져 있다 (Sener 등, 1981). 그래서 TNF-α가 이들 신호 전달 물질을 지속적으로 활성화시키는 Ca\(^{2+}\) Channel에 영향을 미치는지 알아보고자 PKC의 모든 isoform을 저해하는 것으로 알려져 있는 GF109203X과 PKCα, β, γ, δ, ε, ζ, η를 저해하는 Go6983과 PKCα, β, γ를 특이적으로 저해하는 Go6976를 TNF-α와 같이 동시에 24시간 처리해보았으나 감소된 인슐린 분비에는 변화가 없었다. 그리고 PKA의 활성을 억제하는 저해제인 H-89를 처리했을 때에는 인슐린 분비가 TNF-α 단독에 의해 감소된 GSIS보다 오히려 더욱 감소되는 것으로 나타났다 (Fig.16).

TNF-α에 의해 활성화되는 신호 전달물질로 P38, JNK, NFKB, ERK등이 지급까지 밝혀져 있다. 그래서 이들 물질의 저해제인 SB203580, SP600125, SN50, PD98059를 농도별로 10ng/ml의 TNF-α와 동시에 처리하여 24시간
배양 후, GSIS를 조사해보았다. 이 중에서 P38과 ERK, JNK를 각각 저해시켰다
니 감소되었던 GSIS 반응이 평균 10~20% 정도 회복이 되었으며 NFkB를 저해
시켰다니 기저 인슐린 농도는 오히려 감소하는 것으로 나타났다. 하지만 기저 인
슐린 농도에 비해 GSIS의 반응성이 가장 높게 나타났다. 그래서 GSIS 회복률이
가장 높은NFkB가 TNF-α에 의한 GSIS 감소 기작에 중요한 역할을 하는 신호
전달 물질로 생각된다 (Fig.17). TNF-α, IL-1β와 같은 cytokine들은 베타
세포 내에서 NO나 ROS 같은 물질을 만들어내서 베타 세포의 기능이상을 유발
하는 것으로도 알려져 있기 때문에 이들의 관련성을 알아보기 위해서 항산화제인
NAC과 NMMA를처리하여 보았으나 감소된 인슐린 분비에 변화가 없었다
(Fig.18).
Fig. 17. Involvement of PKC and PKA in GSIS inhibition by TNF-α
INS-1 cells were cultured for 24h in medium contained with TNF-α or in medium supplemented with GF109203X (1 μM), Go6976 (1 μM), Go6983 (1 μM), H-89 (20 μM). Following 1h preincubation with buffer containing 0.2mM glucose. GSIS were investigated after 2h incubation.
Fig. 18. Involvement of TNF-α mediated signaling pathway in GSIS inhibition by TNF-α. INS-1 cells were cultured for 24h in medium contained TNF-α with or in medium supplemented with SB203580 (10 μM), SP600125 (20 μM), SN50 (20 μM). following 1h preincubation with buffer containing 0.2mM glucose. GSIS were investigated after 2h incubation.
Fig. 19. Involvement of TNF–α induced ROS and NO in GSIS inhibition by TNF–α INS–1 cells were cultured for 24h in medium contained with TNF–α or in medium supplemented with NAC (2 μM), NMMA (100 μM). following 1h preincubation with buffer containing 0.2mM glucose. GSIS were investigated after 2h incubation.
I. IkBα 유전자 과발현 세포주

(Fig. 18)에서 보았듯이 INS-1 세포에서 TNF-α에 의해 GSIS가 저하되는 것을 NFkB 저해제인 SN 50이 회복시키는 것을 확인하였다. SN 50에 의한 인슐린 분비 회복 효과를 재확인하기 위해서 IkBα 유전자를 과발현시킨 INS-1 세포주를 만들었다. (Fig. 20)에서와 같이 IkBα 유전자를 과발현 시킨 세포주에서 그 단백질량을 Western blot으로 확인하였다. 대조군으로는 pc DNA3를 transfection 시킨 세포를 이용하였다. 여러 clone 세포 중에서 IkBα 단백질 발현량이 많은 4가지 clone 세포를 골랐다. clone #1와 clone #2는 western blot 결과가 예상대로 나타난 반면 clone #3와 clone #4는 IkBα의 단백질 size가 높게 나타났다.
Fig. 20. Establishment of cell lines stably overexpressing IkBα
INS-1 cells were transfected with a cDNA construct encoding IkBα, and stable cell lines were selected. Enhanced expression of IkBα was confirmed by western blot analysis. ①→pcDNA3 ②→clone #1 ③→clone #2 ④→clone #3 ⑤→clone #4
제 2형 당뇨병의 주요 병인 중 하나는 베타 세포의 기능이상에서 비롯된 인슐린 분비 결핍이다. 지속적인 고혈당의 증가가 유리지방산에 의해 생성된 ROS와 같은 free radical과 TNF-α, IL-1β와 같은 세포독성 cytokine들이 인슐린 분비 감소의 원인으로 보고되어있다 (Evans 등, 2003). 제 2형 당뇨병 환자에게서 증가되어있는 Proinflammatory cytokine인 TNF-α (Tumor necrosis factor-α)는 INS-1 베타 세포에서 GSIS를 저해하는 것으로 이미 밝혀져 있다 (Zhang 등, 1995).

본 실험에서는 INS-1 베타 세포에서 TNF-α가 GSIS에 미치는 영향을 조사하였다. (Fig. 4)에서 보는 바와 같이 TNF-α는 농도와 시간에 비례해서 GSIS를 50%이상 감소시켰고 배양액에서 TNF-α 제거 후, 6시간 만에 감소되어있던 GSIS가 정상으로 회복되는 것이 확인되었다. 그렇다면 TNF-α가 포도당 이외의 인슐린 분비 자극 물질들에 의한 인슐린 분비에는 어떤 영향을 미칠 것이지 알아보고자 (Fig. 6, 7, 8)의 실험을 수행하였다. TNF-α가 포도당 이외의 인슐린 분비 자극 물질들에 의한 인슐린 분비에 나타내는 영향에 대해서는 연구결과가 아직 없는 것으로 보인다. 베타 세포에 L-leucine과 Glutamate를 동시에 처리하면 포도당 자극에 대한 1, 2차 인슐린 분비 반응과 유사하게 나타난다 (Sener 등, 1981). (Fig.6)에서 L-leucine과 Glutamate를 각각 또는 동시에 처리하여 보았는데 각각 처리하였을 때 보다 둘을 동시에 처리하였을 때 인슐린 분비 저해 현상이 더욱 뚜렷해 나타났으며 포도당 자극 인슐린 분비 저해 효과와 비슷한 양상을 인슐린 분비가 50%이상 감소하는 것을 확인하였다. (Fig.7)에서는 K_ATP channel의 하위구조 중 하나인 SUR1과 결합하여 인슐린 분비를 일으키는 것으로 알려져 있는 Sulfonlyurea (Glimepiride와 Tolbutamide)에 의한 인슐린 분비와 Kir 6.2에 결합하여 인슐린 분비를 유발하...
는 것으로 알려져 있는 Imidazolin (Phentolamine과 Efaroxan)에 의한 인슐린 분비가 TNF-α에 의해서 감소되었다. L-type Ca\(^{2+}\) channel의 agonist인 Bay K8644는 단독으로 세포 내 Ca\(^{2+}\)량을 증가시켜 인슐린 분비를 일으킨다 (Minami 등, 2002). 그래서 (Fig. 8A)에서 이 약제에 의한 인슐린 분비를 조사 해 보았더니 이 역시 TNF-α에 의해서 인슐린 분비가 50% 정도 감소되었다. 반면 고농도의 KCl에 의한 인슐린 분비는 약간 감소하는 것으로 보인다. 그리고 (Fig. 8C)에서 보듯이 K\(_{ATP}\) channel 독립적으로 인슐린을 분비를 촉진하는 물질인 PMA, Forskolin등을 처리하여 인슐린 분비를 비교하여 보았는데 이들 물질에 의한 인슐린 분비는 TNF-α에 의해 영향을 받지 않았다. 이러한 결과들은 제 2형 당뇨병 베타 세포에서 포도당 자극 인슐린 분비 반응의 감소 모델로서 인슐린 분비의 desensitization현상과 비슷한 것으로 생각되어진다.

그래서 우리는 TNF-α가 포도당 자극 인슐린 분비를 저해하는 작용점과 신호전달과정을 알아보고자 포도당 자극 인슐린 분비과정에 관련된 경로를 크게 4가지로 구분하여 조사해보았다. 첫째 TNF-α가 INS-1 베타 세포 내 인슐린 생합성량에 영향을 주어서 포도당 자극 인슐린 분비 저해를 일으키는지 알아보고자 (Fig. 9)의 실험을 수행하였다. 베타 세포에서 인슐린 생합성이 감소하는 것은 당뇨병에서 인슐린 분비 감소를 일으키는 현상중 하나이다. 이 결과에서 보여주듯이 INS-1 베타 세포에서 TNF-α는 인슐린 유전자 발현량과 세포 내 인슐린 단백질 발현량을 감소시키지 않았다. 이와 같은 결과는 기존에 밝혀진 결과와 동일하였다 (Zhang 등, 1995). 두 번째는 TNF-α가 포도당의 유입으로부터 당당과정을 거쳐 ATP를 생성해내는 대사과정에 미치는 영향을 조사하였다. TNF-α가 베타세포에서 포도당 운반체인 GLUT 2와 해당과정에 중요한 효소인 Glucokinase의 유전자 발현을 억제한다고 보고되어 있다 (Park 등, 1999). 그래서 (Fig.10)에서 이들의 mRNA 유전자 발현량을 확인하여 보았으나 우리의 실험에서는 감소하지 않는 것으로 나타났다. 그리고 포도당이 해당과정을 거쳐 만들어진 ATP/ADP 비율의 증가는 GSIS에 매우 중요하다 (Henquin 등, 1999).
2003). 그래서 세포 내 ATP량을 측정하여 보았다. TNF-α가 Glucokinase의 활성도를 떨어뜨리고 ATP 합성량이 감소되는 것이 GSIS감소의 원인으로 보고 되어있다. (Park 등, 1999). 하지만 우리 실험에서는 TNF-α에 의한 ATP의 양적인 감소는 없는 것으로 조사되었다. 또한 (Fig. 7)의 결과에서 ATP 합성과 상관없이 인슐린 분비를 유발하는 것으로 알려져 있는 Sulfonylurea류 (Tolbutamide와 Glimepiride)와 Imidazolin (Phentolamine과 Efaroxan)에 의한 인슐린 분비가 TNF-α에 의해 감소되는 것으로 보며 ATP 합성량 변화와 GSIS 감소와는 상관이 없는 것으로 생각된다. 셋째로 인슐린 분비에 결정적으로 중요한 역할을 하는 Ca$^{2+}$의 유입량의 변화를 조사해보았다. TNF-α는 단독으로 세포 내 Ca$^{2+}$의 농도를 증가시켰고 (Fig. 12A), (Fig. 12B)의 결과에서 보여주듯이 TNF-α는 INS-1 베타 세포에서의 포도당 자극 Ca$^{2+}$의 유입량과 그 박동성을 현저하게 감소시켰다. MIN 6 베타 세포에서 포도당에 대한 인슐린 분비 반응성이 감소되어 있는 세포의 경우에도 세포 내 Ca$^{2+}$량이 증가되어 있었고 이 Ca$^{2+}$량을 Nifedipine와 Diazoxide로 정상화 시켰을 때 포도당에 대한 인슐린 분비 반응이 회복된다는 연구 결과가 있었다 (Minami 등, 2002). INS-1 베타 세포에서 GSIS에 필요한 외부 Ca$^{2+}$은 L-type Ca$^{2+}$ channel을 통해 유입되며 Nifedipine은 이 channel을 막는 저해제이다 (Liu 등, 2003). 그래서 본 실험에서 세포 내 Ca$^{2+}$유입에 관련된 저해제들 - Nifedipine, Diazoxide, Dantrolene, Xe C-을 이용하여 세포 내 Ca$^{2+}$ 농도를 정상화 시켜보았더니 Nifedipine, Diazoxide에 의해 GSIS가 100% 회복되는 결과를 얻었다. TNF-α는 단독으로 세포 내 Ca$^{2+}$의 농도를 증가시켰으나 TNF-α가 단독으로 인슐린 분비를 일으키지는 못했다. 그 이유는 아직 모르지만 TNF-α에 의한 세포 내 기저 Ca$^{2+}$농도의 증가가 포도당 자극 Ca$^{2+}$ 유입량의 감소와 관련되어 있을 수도 있다. 그리고 (Fig. 8A)에서 Bay K8644에 의한 인슐린 분비가 감소된 것으로 보아 L-type Ca$^{2+}$ channel의 이상이 직접적으로 TNF-α에 의한 인슐린 분비 저해에 관여할 가능성이 있다. 이상의 실험들로 미루어보아 GSIS의 저해 기작은
K<sub>ATP</sub> channel이 닫힘으로서 L-type Ca<sup>2+</sup> channel이 열려서 일어나는 Ca<sup>2+</sup>의 유입에 이상이 있衝으로 생각되어진다.

MIN6 베타 세포에서는 세포 내 Ca<sup>2+</sup> 농도가 지속적으로 증가되어있으면 channel 하위구조의 유전자 및 단백질 발현량이 감소한다고 보고되어 있다 (Minami 등, 2002). 그래서 K<sub>ATP</sub> channel과 L-type Ca<sup>2+</sup> channel의 발현량을 조사하기 위해서 각각의 하위 구조인 Kir 6.2, SUR 1, α1D, β3의 mRNA 발현량과 총 단백질량을 확인해 보았다. (Fig. 14과 15)에서 보듯이 Channel의 유전자 발현량과 총 단백질의 양적 변화는 없는 것으로 나타났다. 그리고 INS-1 베타세포에서도 세포 내 증가된 Ca<sup>2+</sup>이 L-type Ca<sup>2+</sup> channel 하위구조인 α1D의 이동을 조절한다고 보고되어있다 (Huang 등, 2004). 그래서 세포 내 소기관의 막구조에서 세포막으로의 Channel의 이동 여부를 조사해 보았다. (Fig. 16)에서 보듯이 이 역시 변화가 없는 것으로 나타났다. 네번째로 증가한 Ca<sup>2+</sup>으로부터 인슐린 분비가의 과정에 TNF-α의 영향을 조사해 보았다. K<sub>ATP</sub> channel과 독립적으로 즉, Ca<sup>2+</sup>유입량의 변화와는 상관없이 인슐린 분비를 유발하는 PMA, Forskolin과 같은 약제에 의한 인슐린 분비가 정상적으로 이루어진 것으로 미루어 인슐린이 분비되는 insulin secretory machinery에는 이상이 없다고 점검할 수 있었다 (Fig. 8C).

PKA나 PKC는 베타 세포에서 L-type Ca<sup>2+</sup> Channel의 활성도를 조절하는 것으로 보고되어 있다 (Arkhammar 등, 1994). 그래서 TNF-α가 이들 신호 전달 물질을 지속적으로 활성화시켜 L-type Ca<sup>2+</sup> Channel에 영향을 미치는지 알아보고자 각각의 저해제를 사용하여 실험해 보았으나 감소된 GSIS에는 아무런 영향이 없었다 (Fig. 17). (Fig. 18)에서는 TNF-α가 활성화 시키는 신호 전달 물질들을 저해시켜 보았다. TNF-α와 JNK 저해제를 동시에 24시간 처리한 후, GSIS를 조사해 본 결과 감소되었던 GSIS가 대조군에 비해 더욱 증가하였다. 그러나 JNK 저해제 경우에는 그 단독 처리만으로도 기저 인슐린 농도가 두 배 이상 증가하였다. NFkB를 저해시켰을 때는 감소되었던 GSIS가 70~80%정
도 회복되었다. 반면 기저 인슐린 분비는 감소하여 결국 포도당에 대한 인슐린 분비 반응이 JNK를 저해시켰을 때 보다 높게 나타났다. TNF-α에 의해 활성화된 NFkB는 GSIS를 저해하는데 관여하는 것으로 생각된다. TNF-α는 또한 IL-1β와 같은 cytokine들과 같이 베타 세포 내에서 NO나 ROS와 같은 free radical 물질을 만들어내서 베타 세포의 기능이상을 유발하는 것으로도 알려져 있다. 하지만 본 실험의 (Fig. 19)에서는 이러한 이 베타 세포의 기능이상을 일으키는데 영향이 없는 것으로 조사되었다. MIN6 베타 세포에서 cytokine에 의해 유발되는 세포의 기능이상이 NO와 독립적으로 일어난다는 보고도 있었다 (Wu 등, 2001).

MIN6 베타세포에서 탈분극과 세포 내 Ca\(^{2+}\)의 유입에 의해서 NFkB가 활성화되고 (Bernal-Mizrachi 등, 2002) 베타 세포에서 NFkB의 활성도가 GSIS에 필요하다고 보고되어 있다 (Norlin 등, 2005). 그러나 우리의 실험에서는 TNF-α에 의해 활성화된 NFkB를 저해시켰을 때 감소되었던 GSIS가 회복되는 것을 확인하였다. 따라서 TNF-α에 의해 활성화된 NFkB의 활성화가 GSIS를 저해하는데 일부 관여할 것으로 생각된다. 기존 연구 결과에 따르면 제2형 당뇨병 모델 동물인 ZDF 쥐의 췌장 소도세포에서 포도당 자극 Ca\(^{2+}\) 유입량의 감소가 유발되는데 이는 포도당 자극 인슐린 분비 반응의 감소에 중요한 원인이 된다고 보고되어 있다 (Roe MW외, 1996). 결과적으로 우리의 실험에서도 관찰되었던 세포 내 포도당 자극 Ca\(^{2+}\) 유입량의 감소가 INS-1 베타 세포에서 인슐린 분비 반응을 감소시키는 주요 원인으로 생각되어지며 TNF-α에 의해 활성화된 NFkB가 일부 역할을 할 것으로 생각된다. 그러나 TNF-α에 의해 활성화된 NFkB와 TNF-α에 의해 감소된 포도당 자극 Ca\(^{2+}\) 유입량과의 관계는 아직 규명하지 못하였다. 그래서 우리는 NFkB를 활성화 시키는 NIK와 NFkB를 저해하는 IkB를 과발현 시킨 세포주를 구축하여 이를 연구해볼 계획이다. 앞으로 우리의 연구는 제2형 당뇨병 환자에게서 나타나는 인슐린 분비 결핍현상을 이해하는데 도움이 될 것으로 생각된다.
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- ABSTRACT -

Studies of TNF-α–induced GSIS Inhibition in INS-1 β-cell

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Background and aims: Pancreatic β-cell which produce and secrete insulin, is responsible for regulation of plasma glucose concentration. GSIS depends on coordinated glucose uptakes, oxidative metabolism and Ca^{2+}–triggered insulin exocytosis. Impaired GSIS is a hallmark of type II Diabetes. It has been reported that TNF-α, proinflammatory cytokine, was increased in obese type II Diabetes states and it inhibits GSIS in INS-1 β-cell. This mechanism has not been examined in detail. So we investigated the mechanism which TNF-α inhibits GSIS in INS-1 β-cell.

Result: TNF-α suppressed significantly GSIS on dose and time dependent manner. When TNF-α has been removed from medium, the decreased GSIS was recovered completely. Insulinotropic stimuli–aminoacid, sulfonylurea, imidazolin–induced insulin secretion was also decreased about 40–50% by TNF-α (10ng/ml) treatment for 24 hours. Bay K8644, Ca^{2+} channel agonist, induced insulin secretion
decreased about 50% by pretreatment of TNF-α in INS-1 cell. But KCl-induced insulin secretion, evoked by depolarization, was decreased a little. And PMA, forskolin induced insulin secretion was not decreased by TNF-α. This indicates that insulin secretory machinery did not affect by TNF-α.

After pretreatment of TNF-α for 24h, expression of insulin, GLUT2 and GK did not affect. ATP production didn't, neither. This indicates that insulin biosynthesis, glucose uptake and oxidative metabolism have no effect by TNF-α treatment. Glucose stimulated Ca^{2+} influx and Ca^{2+} oscillation was decreased by TNF-α treatment. Both DZX, K_{ATP} channel opener, and Nifedipine, L-type Ca^{2+} channel blocker, markedly improved the decreased GSIS by TNF-α. This indicates that the decrease of Ca^{2+} influx through L-type Ca^{2+} channel could be involved in TNF-α-induced GSIS inhibition. Thus we investigated both expression and trafficking of channels but didn't change.

We tested whether TNF-α activated signal molecule is related in the TNF-α-induced GSIS inhibition. Decreased GSIS was recovered by SN50, NFkB inhibitor. TNF-α-induced NFkB activation is thought to be responsible for TNF-α-induced GSIS inhibition.

**Conclusion:** Glucose and insulinotropic stimuli—aminoacid, sulfonylurea, imidazolin-induced insulin secretion was significantly suppressed by TNF-α. TNF-α inhibited glucos-stimulated Ca^{2+} influx and oscillation through L-type Ca^{2+} channel. The decreased GSIS was recovered by inhibition of NFkB. Thus TNF-α-induced NFkB activation decreased GSIS with abnormality of
glucose-stimulated $[\text{Ca}^{2+}]_i$ influx in INS-1 cell. Our studies indicated that elevation of basal $\text{Ca}^{2+}$ and decrease of glucose induced $\text{Ca}^{2+}$ influx by TNF-$\alpha$ may contribute to insulin secretion deficiency, phathogenesis of type II Diabetes.

Key words: Tumor necrosis factor-$\alpha$ (TNF-$\alpha$), Glucose stimulated insulin secretion (GSIS), $K_{\text{ATP}}$ channel, L-type $\text{Ca}^{2+}$ channel, NFkB
I. INTRODUCTION

Hepatitis B virus (HBV) are small, enveloped DNA viruses that replicate preferentially in liver cells and are involved in hepatocellular carcinoma (Wands and Blum, 1991). HBV has a partially duplex relaxed circular DNA genome, which is replicated by reverse transcription of the RNA genome to produce DNA. The early event of HBV infection is not clearly elucidated yet since the lack of susceptible cell line. Upon infection, released viruses in cytoplasm enter into nucleus after uncoating, then relaxed circular DNA genome is converted to covalently closed circular (ccc) DNA, which serves as the template for transcription of pregenomic RNA (pgRNA). After nuclear export, pgRNA serves as mRNA for capsid (C) and polymerase proteins and as a template for reverse transcription (Ganem and Schneider, 2001). Additionally, pgRNA in cytoplasm is selectively encapsidated into core particles with polymerase (Bartenschlager et al., 1990; Bartenschlager and Schaller, 1992; Hirsch et al., 1990). For encapsidation, HBV polymerase recognizes the epsilon (ε) sequence, the encapsidation signal, of pgRNA. A polymerase-ε ribonucleoprotein (RNP) complex is formed via structural alterations in both pgRNA and polymerase (Beck and Nassal, 1998; Tavis and Ganem, 1996; Tavis et al., 1998). The RNP complex is recognized and encapsidated by dimers of core proteins to form core particles. Reverse transcription occurs inside the core particle. Phosphodiester bonds are observed between the hydroxyl group of Tyr in the TP domain and the first
nucleotide involved in the priming of reverse transcription (Lanford et al., 1997; Weber et al., 1994; Zoulim and Seeger, 1994). Three or two nucleotides are subsequently copied from the bulge region of ε to make the nascent minus-strand DNA. Following the synthesis of nascent minus-strand DNA, this oligomer covalently linked to the polymerase translocates from ε to direct repeat (DR) 1 at the 3’-end of pgRNA, leading to the elongation of minus-strand DNA (Tavis and Ganem, 1995; Tavis et al., 1994). PgRNA template is degraded concomitantly by RNase H activity of HBV DNA polymerase with the elongation of minus-strand DNA. Then plus-strand DNA synthesis is initiated to generate partially double-stranded relaxed circular mature genome (Ganem and Schneider, 2001). Newly assembled, mature core particles containing relaxed circular DNA may enter two different pathways. They can deliver their encapsidated genome into the nucleus of the same cell, leading to the amplification of the episomal nuclear viral cccDNA and the persistent infection of the cell. Alternatively, they can be enveloped with viral surface proteins and intracellular membrane, forming progeny viruses that are released to extracellular space by secretory pathway. At least the latter pathway, the virion secretion, requires genome maturation (Gerelsaikhan et al., 1996). This indicates that there may be some differences between mature and immature core particles. Recent study with permeabilized cell system reported that the difference between immature and mature core particle is the exposed nuclear localization signal (NLS) at C-terminus of core protein. Differently transported empty, immature or mature core particles to nuclear pore complex (NPC) were demonstrated (Rabe et al., 2003).
Because many viruses replicate in nuclei of their host cells, they must have ways to transport their genomes and other components into host nuclei (Whittaker et al., 2000). In the case of HBV, incoming virus as well as newly assembled core particle must be targeted to nucleus to amplify their ccc DNA pool. It has been suggested that active transport processes are involved in these viral movements in cytoplasm, because cytoskeleton and high protein concentrations effectively restrict free diffusion of large molecules (Ruby-Phelps, 2000). It has been demonstrated that many viruses use cytoskeleton for their intracellular trafficking, including entry, assembly and release (Sodeik, 2000).

This study investigates what determines the different intracellular trafficking of HBV core particles according to DNA genome maturation stages. To accomplish this goal, immature and mature core particle are subdivided into four stages: the encapsidation stage in which core particles contain pgRNA only, the initiation stage of minus-strand DNA synthesis with short oligomer of minus-strand DNA, the elongation stage of minus-strand DNA synthesis in which core particles contains minus-strand DNA and pgRNA hybrid, and the final stage of HBV DNA replication with mature core particles with relaxed circular DNA genome. With these core particles, the involvement of cytoskeleton in core particle trafficking or the targeted transfer to nucleus according to the replication stages of core particle were examined.
II. MATERIALS AND METHODS

A. DNA construction

HBV subtype adw R9, 1.3-length of the HBV genome (Blum et al., 1991), was used to generate the partially redundant HBV construct. The 1.3-length of HBV adw R9 construct was cloned into pGEM4Z, yielding pGEM HBV. Next, the 5’-end of HBV (from nucleotides 1418 to 1818) was deleted by PCR-derived mutagenesis. The 3’-end (a total of 225 nucleotides) was additionally deleted by PCR-derived mutagenesis. To express HBV under the cytomegalovirus immediate early (CMV IE) promoter, partially redundant 3.37 kb of HBV was cloned into pcDNA3 (Invitrogen™, Gaithersburg, MD, U.S.A.). For increasing encapsidation efficiency, 55 nucleotides of the pcDNA3 sequence were deleted (Jeong et al., 2000) by PCR-derived mutagenesis. The resulting construct encompassing wild-type (wt) HBV subtype adw R9 was designated pPB (Kim et al., 2004).

To abolish RNase H activity of polymerase, Asp750 of RNase H domain was changed to Val by mutation of single nucleotide using fusion PCR technique. Reverse primer HBV 10 (5’-GAAACTTCTTCAAGGCTTTGATGATT-3’) which targets nts 952 to 981 and a forward mutagenic primer, HBV 71 (5’-GACAGGAATTGACAGTCCGATGAGCTTT-3’) bind to nts 1346 to 1318 was used in one PCR reaction. In other PCR, reverse mutagenic primer, HBV 70 (5’-
ATCGGAACGTGCAATTCTGGTCTCCTCTC-3’) that bind nts 1326 to 1354 and forward primer PC5 (5’-GCATTAGGTGACACTATAG-3’) which target nts 1018 to 999 of pcDNA3 vector, was used. Resulting 0.39 Kb and 0.64 Kb PCR products was combined by fusion PCR. The fusion PCR product was digested with EcoRV and SacII and cloned into the corresponding restriction sites of pPB, yielding a HBV RH D750V mutant.

Tyr 65 (TAT) in the TP domain of polymerase was modified to Phe (TTT) by site-directed mutagenesis using a fusion PCR technique. The oligonucleotides employed were: HBV15 (5’-GGTACTGTAGGAAAGCCCAGTAAAG-3’), a forward mutagenic primer that targets nts 2488 to 2517, and the reverse primer, HBVc (5’-GGTCTCTTTTCGGAGTGTGG-3’) that binds nts 2259 to 2277 of the HBV genome. In another PCR reaction, the oligonucleotides used included reverse mutagenic primer HBV14 (complementary to HBV15), and forward primer HBVPS1 (5’-GAATCCAGGGTTGGGAAC-3’) that binds nts 2907 to 2927 of the HBV genome. The resulting 0.26 kb and 0.44 kb products were combined for fusion PCR. The fusion PCR product was digested with BspEI and BstEII and cloned between the corresponding restriction sites of pPB, yielding the priming-deficient HBV TP Y65F mutant (Kim et al., 2004).

For constructing the RT reaction-deficient RT YMHA mutant of HBV, the conserved YMDD motif was modified to YMHA by site-directed mutagenesis using a fusion PCR technique. The following oligonucleotides were used in the PCR reaction: HBV40 (5’-CTTGGCCCCCAATACCCACAGCATGCTATAAGCTGAAAGC
CAAAC-3’), forward mutagenic primer specific for nts 722 to 765, and reverse primer HBV2 (5’-GCGGGTCACCATATTCTTG-3’), that targets nts 2820 to 2839 of the HBV genome. In another PCR reaction, oligonucleotides used were reverse mutagenic primer, HBV39 (complementary to HBV40) and forward primer HBV9 (5’-GCGAGAGGACGACAGAATTG-3’), which targets nts 1335 to 1354 of the HBV genome. The resulting 1.17 kb and 0.63 kb PCR products were combined for fusion PCR. The fusion PCR product was digested with EcoRI and EcoRV, and cloned into the corresponding restriction sites of pPB, yielding a HBV RT YMHA mutant (Kim et al., 2004).

To obtain a mutant that does not express core protein, GAA for Glu 8 in the core protein was changed to stop codon (TAA) by site-directed mutagenesis using a fusion PCR technique. The oligonucleotides employed were: HBV43 (5’-GTAGCTCCAAATTATTTAAAGGTCAATGTC-3’), a forward mutagenic primer that targets nts 1906 to 1937 of the HBV genome, and the reverse primer, PC3 (5’-GCAGTACATCAAGTGATC-3’) that binds nts 465 to 483 of the pcDNA3 vector. In another PCR reaction, the oligonucleotides used included reverse mutagenic primer HBV42 (5’-GACCCTTATAATTTTGAGCTACTGTG-3’) that targets nts 1912 to 1941, and forward primer HBV15 (5’-GGTACTGTAGGAAAAAAGCCCAATAAAG -3’) that binds nts 685 to 714 of the HBV genome. The resulting products were combined for fusion PCR. The fusion PCR product was digested with HindIII and BspEI and cloned between the corresponding restriction sites of pPB, yielding the C deficient mutant. All constructs were sequenced to
confirm the presence of specific mutations, and to ensure that no extraneous mutations were introduced during PCR.

**B. Cell culture and transfection**

HuH7 hepatoma cell lines were used for the transfection of HBV wt and mutant clones. HuH7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with penicillin and streptomycin, and 10% fetal bovine serum. HBV wt and mutants plasmids were transfected into HuH7 cells on a 10 cm plate or 6 well plate using Lipofectamine (Invitrogen™, Gaithersburh, MD, U.S.A), according to the manufacturer’s instructions.

**C. Isolation of core particles**

Three days after transfection, HuH7 cells were lysed in 1 ml TNE (10 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1 mM EDTA) containing 1% Nonidet P-40. The clarified lysate was adjusted with 10 mM MgCl₂ and 8 mM CaCl₂ solution, and incubated overnight at 37°C with 20 U DNase I (SIGMA, St. Louis, MO) and 60 U micrococcal nuclease (Calbiochem, San Diego, CA). Cytoplasmic core particles were precipitated with 6.5% polyethylene glycol.

**D. RNase protection analysis (RPA)**
To analyze encapsidated pgRNA, core particles were isolated as described above. PgRNA from core particles was extracted following proteinase K (100 µg/ml) and DNase I (20 U) treatment. To prepare riboprobe for RPA, part of the HBV sequence (nts 1805 to 2187) was cloned into pGEM3Zf(+) vector, generating pRPAFD. From this construct, 446 nts of radiolabeled anti-sense probe were synthesized in vitro using SP6 RNA polymerase with α-32P-UTP (specific activity, 800 Ci/mmol). The RPA procedure was performed using the manufacturer’s protocol (RPA II™, Ambion). Protected pgRNA was 369 nts in length.

E. Northern and Southern Blotting

Total RNA (10 µg) was denatured and electrophoresed on a 1 % agarose gel containing formaldehyde, and blotted onto a nylon membrane. RNA on the membrane was hybridized to a 32P-labeled random-primed probe specific for the HBV sequence. To analyze HBV DNA synthesis by Southern blotting, core DNA was extracted, separated by agarose gel electrophoresis, and hybridized to a 32P-labeled random-primed probe specific for the HBV sequence.

F. Western blotting

Isolated core particles were electrophoresed on a 1 % native agarose gel (Koschel et al., 2000). Core particles were transferred to polyvinylidene fluoride
(PVDF) membranes, as described for Northern blot analysis. Immunoblotting was performed using an anti-HBc antibody (DAKO, Glostrup, Denmark). Horseradish peroxidase-conjugated anti-rabbit secondary antibody and ECL (Enhanced Chemical Luminescence) were employed to visualize HBV core particles.

G. Endogenous polymerase assay (EPA)

Isolated core particles were incubated at 37°C overnight with EPA reaction buffer (50 mM Tris-HCl [pH 7.5], 75 mM NH₄Cl, 1 mM EDTA, 25 mM MgCl₂, 0.1 % β-mercaptoethanol, 0.5 % Nonidet P-40) supplemented with 0.5 mM each of dCTP, dGTP, and TTP and 10 µCi α-³²P-dATP (specific activity, 3000 Ci/mmol). ³²P-labeled reaction mixtures were electrophoresed on a 1 % agarose gel and subjected to autoradiography. ³²P-labeled DNA was extracted following the EPA reaction, and separated by 1 % agarose gel electrophoresis.

H. Immunofluorescence assay (IFA)

HuH7 cells were seeded on cover slips and transfected with HBV wt or mutants. Three days after transfection, cells were fixed with 4 % formaldehyde in PBS for 7 min at room temperature, followed by washing with PBS. Cell membranes were permeabilized by 0.075 % Triton X-100 in PBS for 3 min at room temperature, followed by washing with PBS. After blocking with 1 % bovine serum albumin in
PBS, cover slips were incubated with diluted antibodies in blocking solution for 2 hrs at room temperature. For respective immunofluorescence staining, 1:700 diluted rabbit anti-HBcAg (DAKO, Glostrup, Denmark) for core particles, 1:100 diluted mouse anti-α-tubulin (Calbiochem, San Diego, CA) for microtubule (MT), 1:5000 diluted mouse anti-NPC (Covance) for NPC, or 1:50 diluted mouse anti-vimentin (Abcam) for intermediate filament, were used. After washing the first antibodies, 1:100 diluted fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG or 1:200 diluted rhodamin-conjugated anti-rabbit IgG, the secondary antibodies, were employed for 1 hr at room temperature. The nuclei of cells were stained with 1:50000 diluted 4′,6′-diamidino-2-phenylindole (DAPI). The immunostained cells were visualized under the zeiss LSM 510 confocal laser-scanning microscope.
III. RESULTS

A. Construction of HBV mutants that represent various stages of HBV replication

To obtain the core particles, which represent the various stages of HBV DNA replication, several HBV mutants were constructed in HBV adw R9 wt background (Fig. 1) (Kim et al., 2004). C deficient mutant is defective in core particle formation since core protein synthesis is blocked, but can form RNP complexes. RT YMHA, reverse transcription deficient, can encapsidate pgRNA but DNA replication is completely blocked by mutation of YMDD reverse transcriptase motif to YMHA (Bartenschlager et al., 1990; Bartenschlager and Schaller, 1992; Chang et al., 1990; Hirsch et al., 1990; Radziwill et al., 1990; Roychoudhury et al., 1991). So this mutant makes core particle containing only pgRNA. Priming deficient TP Y65F mutant core particles can synthesize oligomer, the nascent minus-strand DNA, from encapsidated pgRNA (Kim et al., 2004). In this mutant, Tyr 65 in TP domain, responsible for priming reaction, of HBV DNA polymerase was changed to Phe. RH D750V mutant core particles contain pgRNA-minus strand DNA hybrid. Since RNase H activity is defective by mutation of aspartic acid 750, the RNase H active site, to valine in HBV DNA polymerase, pgRNA remain intact after minus-strand DNA synthesis. Thus the replication is arrested at this stage (Radziwill et al., 1990). HBV wt core particles can
make mature relaxed circular DNA genome. It should be noted that various stages of replicative intermediates and pgRNA should be detected from HBV wt core particles. Also, from RH D750V mutant core particles, pgRNA and pgRNA-minus strand DNA hybrid should be detected.

B. HBV RNA expression and core particle formation

HBV RNA expression was analyzed from wt or mutants-transfected HuH7 cells. Total RNA was analyzed by Northern blot analysis. Upon transfection, pgRNA should be expressed by the action of the CMV promoter. Subgenomic RNA sequences should be synthesized under the control of their authentic promoters. The 3.5 kb pgRNA, and 2.1 and 2.4 kb mRNA of surface proteins were detected from wt or mutants- transfected cells (Fig. 2A). There were no significant differences in the RNA expression levels between wt and mutants-transfected cells (Fig. 2A).

Core particle formation was examined by native agarose gel eletrophoresis and Western blotting, using the anti-HBc antibody (Koschel et al., 2000). Core particles were detected from wt and mutant-transfected HuH7 cells except C deficient mutant and showed similar mobility on a native agarose gel (Fig. 2B). C deficient mutant cannot form core particle since core protein expression is blocked.
Fig. 1. Schematic diagram of HBV wild type (wt) and mutant constructs. To obtain HBV core particles at various stages of HBV replications, several HBV mutants were constructed in HBV adw R9 wild-type (wt) background. PgRNA is transcribed under the control of a CMV IE promoter. HBV sequences are shown as thick line and ε sequences on 5’- and 3’-ends are marked. The ORFs of core (C), polymerase (P), surface (S) and X genes are shown as open boxes. Each domain of polymerase (TP, spacer, RT and RNase H) was indicated. The CMV promoter is shown as open arrow.
Fig. 2. HBV RNA expression and core particle formation. (A) Northern blot analysis to detect the expressed HBV mRNA. Total RNA was extracted and separated by 1 % formaldehyde gel electrophoresis, transferred to nylon membranes, hybridized with random-primed $^{32}$P-labeled HBV specific probes, and subjected to autoradiography. PgRNA (3.5 kb) and 2.1 and 2.4 kb mRNA for surface proteins (S) are indicated. (B) Western blot analysis to detect HBV core particles from a native agarose gel. Isolated core particles were transferred to PVDF membranes and incubated with rabbit anti-HBc antibody. HRP-conjugated secondary antibody and ECL were used to visualize core particles. HBV wt and all the mutants, except the C deficient mutant, have core particles.
C. HBV pgRNA encapsidation

HBV pgRNA encapsidation was investigated in HBV wt and mutants. Encapsidated pgRNAs were isolated from wt and mutants core particles and then, analyzed with RPA. Wt, RH D750V, TP Y65F and RT YMHA mutants encapsidated pgRNA (Fig. 3), while the C deficient mutant was unable to encapsidate pgRNA, as expected (Fig. 3).

D. HBV DNA replication

To investigate HBV DNA replication stages of HBV wt and mutants, cytoplasmic core particles were isolated from HBV wt or mutants transfected HuH7 cells. Following extraction of HBV DNA from isolated core particles, Southern blot analysis was performed to detect replicating HBV DNAs. HBV DNA was observed from wt and RH D750V mutant core particles (Fig. 4). HBV wt core particles contain single-stranded, double-stranded, and partially double-stranded relaxed circular HBV DNA. While RH D750V mutant contains some smeared HBV DNA band, which is smaller than double-stranded but larger than single-stranded DNA (Fig. 4). Since RNase H activity of HBV polymerase is defective in RH D750V mutant, this smeared DNA band is believed as pgRNA and minus-strand DNA hybrid. HBV DNA replication was not detected in TP Y65F, RT YMHA or C deficient mutant-transfected cells (Fig. 4). Theoretically, the C-deficient mutant is a dead virus.
Mutation of the conserved reverse transcriptase motif YMDD to YMHA results in a dead virus that does not support HBV DNA synthesis in vivo (Chang et al., 1990; Hirsch et al., 1990). TP Y65F mutant can synthesize short oligomer, but this oligomer is not detected by Southern blotting because short oligomer must be lost from conventional DNA purification process from core particle (Kim et al., 2004).

To confirm the short oligomer synthesis by priming deficient, TP Y65F mutant, EPA was performed by which isolated wt and mutant core particles were incubated with $^{32}$P-dATP. Through EPA reaction, synthesized HBV DNA inside core particle was labeled with $^{32}$P-dATP. After the EPA reaction, labeled core particle and free $^{32}$P-dATP in reaction mixtures were electrophoresed on 1 % native agarose gels, which remove free labeled-nucleotide. Then core particles with radioactively labeled DNA or oligomers were visualized by dry gel autoradiography. HBV wt or RH D750V core particles were labeled with strong intensity and this result is consistent with Southern blot data (Fig 5A). As previously reported (Kim et al., 2004), TP Y65F mutant core particles were weak compared to wt (Fig 5A) which indicated the synthesized short oligomer. In C deficient or RT YMHA mutant, radioactively labeled core particle was not detected (Fig 5A). Upon extracting $^{32}$P-labeled DNA from core particles after EPA, HBV wt and RH D750V mutant DNA displayed the comparable electrophoretic patterns with Southern blot analysis since single-stranded, double-stranded, and partially double-stranded relaxed circular HBV DNA and pgRNA and minus-strand DNA hybrid from HBV wt and RH D750V, respectively. And HBV DNA was not detected in C deficient and RT YMHA mutants (Fig. 5B). This also is in agreement with Southern blot analyses (Fig 4).
Fig. 3. HBV pgRNA encapsidation. RNase Protection Assay to detect encapsidated pgRNA. *In vitro* transcribed radiolabeled antisense RNA probe (446 nts) was hybridized overnight at 50°C with extracted pgRNA from isolated core particles. Protected pgRNA (369 nts) following RNase digestion was run on a 5% polyacrylamide-8M urea gel and visualized by autoradiography.
Fig. 4. Southern blot analysis. Southern blot analysis to detect HBV DNA replication. HBV DNA was extracted from isolated core particles, separated, transferred to nylon membranes, hybridized with a random-primed $^{32}$P-labeled HBV specific probe, and subjected to autoradiography. Single-, double-stranded, and partially double-stranded relaxed circular forms of HBV DNA are marked as ssDNA, dsDNA, and RC, respectively.
**Fig. 5. Endogenous Polymerase Assay (EPA).** (A) Isolated core particles were incubated with EPA reaction buffer supplemented with 0.5 mM each of dCTP, dGTP, TTP and 10 μCi α-32P-dATP (3000Ci/mmol) at 37 °C for overnight incubation. Reaction mixtures were electrophoresed on 1 % agarose gel and subjected to autoradiography. (B) After EPA reaction, 32P-labeled DNA was extracted, separated by 1 % agarose gel electrophoresis and subjected to autoradiography. Single-, double-stranded, and partially double-stranded relaxed circular forms of HBV DNA are marked as ssDNA, dsDNA, and RC, respectively.
E. Intracellular trafficking of HBV core particle in various stages of DNA replication

In above section, replication arrest point was confirmed from each of HBV mutants that represent various stages of HBV DNA replication. To analyze intracellular trafficking according to different DNA maturation stages of assembled HBV core particles, immunofluorescence assay was conducted and visualize by confocal microscope.

After viruses enter into cytoplasm upon infection, many viruses interact with microtubule (MT) to move through the cytoplasm to the vicinity of the nucleus (Bailey et al., 2003; Gilbert et al., 2003; Kelkar et al., 2004; Ogawa-Goto et al., 2003). So, it is presumed that HBV uses MT for intracellular movement. At first, HBV wt-or mutant-transfected into HuH7 cells then core particle and MT were immunofluorescence stained to examine the co-localization between core particle and MT (Fig. 6A). HBV core particles in red were predominantly observed in cytoplasm of wt-and mutant-transfected cells excluding C deficient mutant. HBV wt and all mutant core particles showed similar intracellular distribution, however only HBV wt or RH D750V core particles containing relaxed circular double-strand genome or pgRNA-minus-strand DNA hybrid, respectively, were co-localized with MT. This result suggests that HBV core particle uses MT of host cell for movement through cytoplasm like many other viruses. In addition, it indicates that all core particles that co-localize with MT for transport, completed minus-strand DNA.
To investigate core particle transport toward nucleus, HBV core particles and NPC were immunofluorescence stained. Interactions of core particles and NPC were observed from HBV wt and RH D750V mutant-transfected cells but not from other mutant-transfected cells (Fig. 6B). This result suggests that only core particles in late stages of HBV DNA replication can move to NPC.

From these IFA data, it is reasonable to think that HBV core particles with mature HBV genome or completed minus-strand DNA, may interact with MT and move toward nucleus by MT mediated active transport mechanism and then may associate with NPC to enter nucleus. Since MT network is originated from MT organizing center (MTOC) in peri-nuclear region, HBV core particle that arrive to MTOC may use another way to move to NPC.

To test this speculation, HBV core particles and vimentin, the intermediate filament, were immunofluorescence stained. Fig. 6C showed that vimentin was observed around the nucleus. Also, vimentin were co-localized with HBV wt-and RH D750V mutant-core particles but not with other mutants (Fig. 6C). In addition, this result indicates that vimentin, the intermediate filament, may fill the blank between MTOC and NPC for core particle transport.
A. Localization of core particles and microtubules

**Fig. 6. Intracellular trafficking of HBV core particles.** HuH7 cell were transfected with HBV wt or mutants. By indirect immunofluorescence assay, intracellular HBV core particles were immunostained together with microtubules (A), NPC (B), or vimentins (C) and visualized with confocal laser scanning microscopy. HBV core particle is shown as red while microtubule, NPC and vimentin as green. Yellow signal represents the co-localization of the red and the green.
B. Localization of core particles and nuclear pore complexes
C. Localization of core particles and intermediate filaments

HBV wt  

RH D750V  

TP Y65F  

RT YMHA  

C def
IV. DISCUSSION

Cytoplasmic MT has been evolved for intracellular trafficking of vesicles, organelles, and proteins. Also it has been known that MT are involved in the transport of many viruses (Fuchs and Yang, 1999; Ploubidou and Way, 2001; Sodeik, 2000). Intermediate filaments are needed for mechanical integration of the cytoplasm in higher eukaryotes, and impart intracellular mechanical strength. Several studies reported that vimentin, one of the components of intermediate filament, was shown to interact with viruses or viral proteins and be reorganized in virus replication process (Birkenbach et al., 1989; Chen et al., 1986; Lake et al., 2003; Mora et al., 1987; Nedellec et al., 1998). Although early steps of hepadnavirus infection and viral trafficking are poorly characterized, recent study reported that cytoplasmic trafficking of DHBV viral particles and emergence of nuclear cccDNA require MT for specific time periods during entry (Funk et al., 2004). From these backgrounds, it is presumed that HBV also use host cytoskeleton for movement of core particles through host cytoplasm after core particle assembly as well as during entry for infection.

The HBV virion release from hepatocyte must be tightly regulated events. The current dogma is that the only core particles with mature hepadnavirus genome are preferentially exported from the intracellular compartment (Gerelsaikhan et al., 1996; Perlman and Hu, 2003; Wei et al., 1996). This means that core particles with mature
genome are ready for envelopment. This readiness for envelopment may be applied to nuclear targeting for core particle recycling. And previous report showed the differences in nuclear import and genome release between the immature and the mature core particles (Rabe et al., 2003).

In this study, four different replicative stages of core particles were established. Each replicative stage is the encapsidation stage of core particles which contain pgRNA only, the initiation stage of core particle which contain pgRNA and short oligomer of minus strand DNA, the elongation stage of core particles which contain minus-strand DNA and pgRNA hybrid, and the mature stage of core particles which contain relaxed circular DNA genome. With series of mutants arrested at different DNA maturation stages of core particles, intracellular trafficking of core particles was investigated focusing on retrograde movement for core particle recycling.

Core particles were observed predominantly in cytoplasm, regardless of replication stages in this study. Predominant cytoplasmic expressions of HBV core particles are observed in actively proliferating (Ozer et al., 1996; Serinoz et al., 2003). The experimental system in this study is actively proliferating HuH7 hepatoma cell line in which HBV was expressed and replicated. Thus this may explain predominant cytoplasmic distribution of core particle.

From series of replicative stages of core particles, only core particle in late stages of HBV DNA replication with completed minus-strand DNA or relaxed circular DNA genome, can co-localize with MT, vimentin and NPC of host cells. These results suggest that HBV core particles use MT and intermediate filament for
transport through cytoplasm and may bind to NPC for nuclear import. From these results, the model for retrograde transport of core particles can be proposed. In this model, HBV core particles with mature genome or completed minus-strand DNA interact with MT and move toward nucleus by MT mediated active transport mechanism. Since MT network is originated from MT organizing center (MTOC) in peri-nuclear region, HBV core particle in peri-nuclear region may use another way, intermediate filament, to move to NPC. Then, core particles associate with NPC to enter nucleus.

In addition, core particles with completed minus-strand DNA can be speculated to be ready for nuclear targeting like HBV wt mature core particles with relaxed circular DNA genome, since the core particles with pgRNA-minus-strand DNA hybrid showed similar intracellular localization like wt. This study shows the intracellular transport of RNase H mutant core particles. The result is consistent with previous report that RNase H mutant virus was secreted as virions (Gerelsaikhan et al., 1996; Perlman and Hu, 2003). Taken together, it is believed that core particles undergo some distinctive changes from immature core particle to mature core particle after minus-strand DNA synthesis is completed to move back into nucleus or to get envelope from intracellular membrane.

The factors for different phenotype between immature and mature core particles are still ambiguous. However, from duck hepatitis B virus (DHBV) and HBV model, core particle trafficking to release genome into nucleus is regulated by phosphorylation of core particle (Kann et al., 1999; Kock et al., 2003). Since genome
maturation is correlated with core particle phosphorylation (Rabe et al., 2003), core particle phosphorylation status is probably one of the factors for different trafficking while DNA replication proceeds.

This study showed different intracellular trafficking of HBV core particles and the involvement of cytoskeleton in core particle trafficking or the targeted transfer to nucleus according to DNA genome maturation stages of core particle, with series of replicative stages of core particles.
In this study, the differences in intracellular trafficking of HBV core particle between DNA genome maturation stages were investigated. To accomplish this goal, immature and mature core particles are subdivided into four stages: the encapsidation stage in which core particles contain pgRNA only, the initiation stage of minus-strand DNA synthesis with short oligomer of minus-strand DNA, the elongation stage of minus-strand DNA synthesis in which core particles contains minus-strand DNA and pgRNA hybrid, and the final stage of HBV DNA replication with mature core particles with relaxed circular DNA genome. With this core particles, which cytoskeleton involved in core particle trafficking or which stage of core particle can target nucleus was examined.

As a result, the core particles in late stages of HBV DNA replication with completed minus-strand DNA or relaxed circular DNA genome are co-localized with MT, vimentin and NPC. These results suggest that HBV core particles use MT and intermediate filament to move through host cytoplasm, and can interact with NPC for nuclear transport. From these results, it is reasonable to speculate that HBV core particles with mature genome or completed minus-strand DNA can interact with MT and move toward nucleus by MT-mediated active transport mechanism. To move toward nucleus from peri-nuclear region, MTOC, core particles may use intermediate filament and then associate with NPC to enter nucleus. In addition, core particles
with completed minus-strand DNA can be speculated to be ready for nuclear targeting like HBV wt mature core particles with relaxed circular DNA genome, since the core particles with pgRNA-minus-strand DNA hybrid showed similar intracellular localization like wt.
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다양한 복제단계에서 B형 간염 바이러스의 세포 내 이동

 아주대학교 대학원 의학과
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B형 간염 바이러스의 복제는 새로 형성된 캡시드 입자 내에서 일어난다. 캡시드에 싸여 들어간 pgRNA는 DNA 중합효소에 의하여 음성 가닥의 DNA로 역전사 되고, 동시에 DNA 중합효소의 RNase H 작용에 의하여 분해된다. 그 후 양성 가닥의 DNA가 합성되면서 부분적 이중가닥의 환형 DNA 게놈이 완성된다. 이러한 HBV DNA의 복제가 진행됨에 따라 미성숙 캡시드 입자가 성숙한 캡시드 입자가 되며, 성숙한 캡시드는 세포내의 바이러스의 게놈을 증폭시키기 위하여 다시 핵으로 이동하거나 또는 바이러스 입자로 세포밖으로 방출되기 위하여 세포내 막으로 이동하여 바이러스 외피를 얻는다. 이 연구에서는 HBV DNA의 복제가 진행됨에 따른 캡시드 입자의 세포 내 이동을 조사하였다. 다양한 HBV DNA 복제 단계에 있는 캡시드 입자를 얻기 위하여 몇 개의 HBV 돌연변이주를 만들었다. RT YMHA 변이주는 역전사 결손 변이주로 캡시드 내에서 pgRNA가 역전사 될 수 없다. DNA 복제 시동 결손 변이주인 TP Y65F 변이주의 캡시드 입자는 새로 합성된 짧은 가닥의 음성 가닥 DNA를 포함하고
있다. RH D750V 변이주는 DNA 중합효소의 RNase H 작용 결손이므로 캡시드 내에 pgRNA와 음성가닥의 DNA로 이루어진 혼성 가닥을 갖고 있다. HBV wt의 캡시드는 완성된 부분적 이중가닥의 환형 DNA 게놈을 갖고 있으며, 다양한 복제 매개체를 갖는 캡시드도 포함되어있다. 이들 변이주를 HuH7 세포에 발현 시킨 후 면역형광법으로 캡시드 입자의 분포를 조사하였다. 그 결과 DNA 복제 단계에 따라 캡시드의 분포에 차이가 있는 것을 확인하였다.

핵심어: B형 간염 바이러스의 캡시드 입자, 복제단계, 세포 내 분포
I. INTRODUCTION

Hepadnaviruses are small, enveloped DNA viruses that replicate preferentially in liver cells and are involved in hepatocellular carcinoma (Wands and Blum, 1991). HBV, a prototype of hepadnavirus, has a partially duplex relaxed circular DNA genome, which is replicated by reverse transcription of the RNA genome to produce DNA.

Assembly of replication competent HBV nucleocapsid requires interaction of core protein, polymerase and pgRNA of HBV (Hatton et al., 1992). The core protein consists of 183 or 185 amino acid and assemble into core particle via dimeric intermediates (Zhou and Sandring, 1992). The N-terminal portion of core protein (amino acids 1-144) participates in capsid particle assembly by protein-protein interaction and is, by itself, assembly competent (Birnbaum and Nassal, 1990; Gallina et al., 1989). The C-terminal portion of core protein contains protamine-like domain, which is rich in arginine residue, and is known as nucleic acid binding domain. Although C-terminal domain of core protein is dispensable for capsid particle assembly, this domain is involved in viral replication including pgRNA encapsidation (Beames and Lanford, 1993; Birnbaum and Nassal, 1990; Gallina et al., 1989; Nassal, 1992; Pogam et al., 2005). Although many researchers suggest the importance of C-terminal domain of core protein in hepadnavirus pgRNA encapsidation and DNA replication with series of C-terminally truncated mutants,
critical amino acid residues or motif for pgRNA encapsidaion or DNA replication has not been demonstrated yet.

In many studies, chimeric viruses, such as retrovirus or avian hepadnavirus, were used to identify the function of protein domains or cis-acting elements of viruses (Berkowitz et al., 1995; Certo et al., 1999; Kaye and Lever, 1998; Kristin et al., 2004). In this study, to identify the nucleic acid binding domain for HBV replication, chimeric core proteins of HBV were constructed by exchanging the HBV core protein C terminal region with corresponding region of DHBV core protein. DHBV core proteins that consist of 262 amino acids can form a three-dimensional nucleocapsids structure similar to that of HBV (Kenney et al., 1995). Chimeric core proteins were designed to contain various lengths of corresponding DHBV sequences, while N-terminal sequences of HBV core protein were unchanged. With these chimeric core proteins, HBV pgRNA encapsidation and HBV DNA replication were examined. This chimeric core protein study shows that chimeric core protein can trans-complement HBV core proteins for C deficient mutant. This study also shows that the 45.2% of amino acid sequence homology at core protein C-terminal region for nucleic acid binding domain is sufficient to encapsidate HBV pgRNA and synthesize HBV DNA.
II. MATERIALS AND METHODS

A. HBV plasmid DNA construction

HBV subtype adw R9 plasmid construct, pPB, was used as a wild-type (wt) construct (Kim et al., 2004).

C deficient mutant that do not express core protein was generated by introducing stop codon (TAA) to Glu 8 (GAA) of core protein by site-directed mutagenesis using a fusion PCR technique (described in part 1). This C deficient mutant expresses pgRNA and other HBV proteins except core protein. This C deficient mutant will be used throughout the experiments to test the trans-complementation of chimeric core protein for HBV encapsidation and DNA replication.

Plasmid construct providing HBV core protein was generated which contains HBV core ORF and HBV post-transcriptional regulatory element (PRE). pPB was digested with BstEII and subject to blunt end reaction with T4 DNA polymerase. After this product was digested with EcoRV, two blunt ended BstEII and EcoRV recognition site were ligated, resulting deletion between nts 2824 and 1045 and named as pεHCP. The ε sequence was additionally deleted by PCR technique. The HindIII recognition site containing reverse primer HBV 41 (5’-AGAGGGGAAGCTTGTGGCTTTGGGGCATG-3’) targeting nts 1890 to 1905 and forward primer
HBV 15 (5’-GGTACTGTAGAGGAA TAAAGCCCAGTAAAG-3’) targeting 2517 to 2488 was used in PCR reaction. Resulting 0.63 Kb PCR product was digested with HindIII and BspEI, followed by cloning into the corresponding restriction site of pεHCP, resulting ε sequence deletion and named as pHCP.

To generate plasmid construct providing DHBV core protein which contains DHBV core ORF and HBV PRE, DHBV core gene of pCMVDHBV were cloned into pcDNA3 between HindIII and EcoRV enzyme site, yielding pDC. To add HBV PRE sequence to pDC, at 3’ end of DHBV core gene, pDC was digested with XcmI and subject to blunt end reaction with T4 DNA polymerase, then digested with ApaI. HBV PRE sequence from EcoRV to ApaI of pPB was cloned into pDC between blunt ended XcmI and ApaI recognition sites, yielding pDCP.

Core protein chimera HD 192-262 was constructed by PCR derived recombination of HBV and DHBV gene. In brief, pDCP was PCR amplified using reverse primer HBV90 (5’- ATCAACACTTCCGGAACCAATCCAGGTGGCTC AGGGAGG-3’) targeting nts 2322 to 2337 of HBV core gene and nts 199 to 221 of DHBV core gene, and forward primer HBV19 (5’-GTGCGCAGACCAA TTTAGCTA TGGGAGAAG-3’) targeting nts 1789 to 1808 of HBV gene. Resulting 1.2 Kb of product was digested with BspEI and SacII, then cloned into pHCP between corresponding restriction enzyme site.

Core chimera HD 221-262 was constructed by PCR derived recombination. The primer employed were HBV91 (5’-ATCAACACTTCCGGAACCAACACAGTTG TCTATGGGAGAAG-3’), reverse primer targeting nts 2322 to 2337 of HBV core
gene and nts 286 to 308 of DHBV core gene, and forward primer HBV19 described above. Resulting 1 Kb of PCR product was digested with BspEI and SacII then cloned into pHCP between corresponding restriction enzyme site.

Core chimera HD 192-220 was constructed by fusion PCR technique. pDCP was PCR amplified using reverse primer HBV90 described above and forward primer HBV93 (5’-CAACAGTAGTTTTAACTTTTCTTTCTTAGGTTCC-3’) that bind to nts 2338 to 2347 of HBV core gene and 262 to 285 of DHBV core gene. In another PCR reaction, pHCP was PCR amplified using reverse primer HBV92 (5’-AAAAGTTAAAAACTA GTGGTTAGACGACGGGAC-3’) that bind to nts 2338 to 2361 of HBV core gene and nts 276 to 285 of DHBV core gene, and forward primer HBV19 described above. The resulting 0.3 Kb and 0.9 Kb of PCR products were combined by fusion PCR. The fusion PCR product was digested with BspEI and SacII and cloned between the corresponding restriction sites of pHCP. All constructs were sequenced to confirm the presence of specific mutations, and to ensure that no extraneous mutations were introduced during PCR.

B. Cell culture and transfection

HuH7 hepatoma cell lines were used for the transfection of HBV wt, mutant clones, and various core constructs. HuH7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with penicillin and streptomycin, and 10 % fetal bovine serum. 8 µg of HBV wt plasmid was transfected, or 2 µg of C deficient
mutant and 6 μg of various core proteins construct plasmids were co-transfected into HuH7 cells on a 10 cm plate using Lipofectamine (Invitrogen™, Gaithersburh, MD, U.S.A.), according to the manufacturer’s instructions.

C. Isolation of core particles

Three days after transfection, HuH7 cells were lysed in 1 ml TNE (10 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1 mM EDTA) containing 1 % Nonidet P-40. The clarified lysate was adjusted with 10 mM MgCl₂ and 8 mM CaCl₂ solution, and incubated overnight at 37°C with 20 U DNase I (SIGMA, St. Louis, MO) and 60 U micrococcal nuclease (Calbiochem, San Diego, CA). Cytoplasmic core particles were precipitated with 6.5 % polyethylene glycol.

D. RNase protection analysis (RPA)

To analyze encapsidated pgRNA, core particles were isolated as described above. PgRNA from core particles was extracted following proteinase K (100 μg/ml) and DNase I (20 U) treatment. To prepare riboprobe for RPA, part of the HBV sequence (nts 1805 to 2187) was cloned into pGEM3Zf(+) vector, generating pRPAFD. From this construct, 446 nts of radiolabeled anti-sense probe were synthesized in vitro using SP6 RNA polymerase with α-³²P-UTP (specific activity, 800 Ci/mmol). The RPA procedure was performed using the manufacturer’s protocol.
(RPA II™, Ambion). Protected pgRNA was 369 nts in length.

E. Northern and Southern Blotting

Total RNA (10 µg) was denatured and electrophoresed on a 1 % agarose gel containing formaldehyde, and blotted onto a nylon membrane. RNA on the membrane was hybridized to a $^{32}$P-labeled random-primed probe specific for the HBV sequence. To analyze HBV DNA synthesis by Southern blotting, HBV DNA was extracted from core particle, separated by agarose gel electrophoresis, and hybridized to a $^{32}$P-labeled random-primed probe specific for the HBV sequence.

F. Core particle Western blotting

Isolated core particles were electrophoresed on a 1 % native agarose gel (Koschel et al., 2000). Core particles were transferred to polyvinylidene fluoride (PVDF) membranes, as described for Northern blot analysis. Immunoblotting was performed using an anti-HBc antibody (DAKO, Glostrup, Denmark). Horseradish peroxidase-conjugated anti-rabbit secondary antibody and ECL (Enhanced Chemical Luminescence) were employed to visualize HBV core particles.
III. RESULTS

A. Core protein and chimeric core protein construction

To investigate the nucleic acid binding domain of HBV core protein for HBV replication, chimeric core proteins were constructed by the exchanging the C-terminal region of HBV core protein with corresponding region of DHBV. HBV and DHBV core protein expression plasmids were constructed first naming pHCP and pDCP, respectively, and used as backbone for chimeric core protein constructions. Chimeric core proteins were designed to contain various lengths of corresponding C-terminal region of DHBV sequences, while N-terminal 145 amino acids sequences were unchanged. Amino acid sequence comparison of HBV and DHBV core protein show 26.7% of amino acid sequence homology in whole core protein, while C-terminal domain has 45.2% homology. And DHBV core protein has additional 29 amino acids, which are absent in HBV core protein (Fig. 1A). HD192-262 chimeric core protein construct has N-terminal of HBV core and C-terminal of DHBV core including additional 29 amino acids. HD 192-220 chimeric core protein construct has entire HBV core protein sequence but additional 29 amino acids are inserted between amino acid 145 and 146 of HBV core protein. HD 221-262 chimeric core protein construct with HBV N-terminal and DHBV C-terminal without 29 amino acids was combined. Transcription of all the core protein constructs is controlled by a CMV IE
promoter, and the nuclear export of core RNA is facilitated by HBV post-transcriptional regulatory element (PRE) sequence (Huang and Liang, 1993) (Fig. 1B). As a positive control, HBV subtype adw R9 (Blum et al., 1991) wt construct was used in which transcription of pgRNA is controlled by a CMV IE promoter (Kim et al., 2004). Core protein deficient mutant (C deficient) and various core protein chimeras were co-transfected into HuH7 hepatoma cell line to allow core particle assembly (Fig. 1C). In this experimental system, core proteins were supplied in trans from chimeric core protein constructs, and pgRNA and other HBV proteins were provided from C deficient mutant. HBV core, pHCP, was used as another positive control to trans-complement the pgRNA encapsidation of C deficient mutant, from which core proteins are supplied in trans (Fig. 1C). Whether the various chimeric core proteins trans-complement the pgRNA encapsidation of C deficient mutant was tested.

B. HBV RNA transcription

Initially, HBV RNA expression was analyzed in wt transfected, or C deficient mutant and core protein plasmids co-transfected HuH7 cells. Total RNA was analyzed by Northern blot analysis. Upon transfection, pgRNA was expressed by the action of the CMV promoter. Subgenomic RNA sequences were synthesized under control of their authentic promoters. The 3.5 kb pgRNA, 2.1 and 2.4 kb mRNA of surface proteins were detected from wt transfected, and core constructs and C
deficient mutant co-transfected cells (Fig. 2). There were no significant differences in the RNA expression levels.
Fig. 1. Schematic diagram of core protein and chimeric core protein constructs.

(A) The Amino acid sequences alignment of C-terminal domain of HBV and DHBV core protein. The yellow represents identical sequences between HBV and DHBV and the green represents similar amino acids. Phosphorylation sites are marked with asterisks. (B) Core and chimeric core proteins for trans-complementation. HBV and DHBV sequences are depicted as blue and red, respectively, and the ε sequences at 3’-ends are marked. The C gene ORF is presented as closed boxes. CMV IE promoter is represented as an open arrow. PRE is post-transcriptional regulatory element. (C) HBV full genome constructs. The overlapping ORF of C, P, S and X genes are presented as open boxes. Each domain (TP, spacer, RT and RNase H) of the polymerase is indicated. Mutant constructs in a wt HBV adw R9 background which is unable to express core protein, are designated as C deficient. The positions of core point mutations are indicated as closed arrowheads.
Fig. 2. **HBV RNA expression.** Northern blot analysis to detect HBV mRNA expression from C deficient mutant and various core expressing constructs co-transfected HuH 7 cells. Total RNA was extracted and separated by 1% formaldehyde gel electrophoresis, transferred to nylon membranes, hybridized with random-primed \(^{32}\)P-labeled HBV specific probes, and subjected to autoradiography. pgRNA (3.5 kb), 2.1 and 2.4 kb mRNA for surface protein(S) are indicated.
C. Chimeric core particle assembly

To investigate assembly competency of chimeric core proteins, core particle formation was examined by native agarose gel electrophoresis and Western blotting using the anti-HBc antibody (Koschel et al., 2000). Core particles were detected from two positive controls and from C deficient mutant and either HD 192-220 or HD 221-262, chimeric core protein mutants, co-transfected HuH7 cells. In addition, two chimeric core particles, assembled from HD 192-220 or HD 221-262 chimeric core proteins, displayed similar mobility with wt core particles on a native agarose gel with HBV wt. Because anti-HBV core antibody was used to detect various core particles, DHBV core particle may not detected by anti-HBc antibody. However HD 192-262 chimeric core protein cannot form core particle (Fig. 3). These results show that even though chimeric core proteins were designed to contain intact N-terminal assembly domain of HBV, C-terminal substitution to DHBV can affect core particle assembly.

D. HBV pregenomic RNA encapsidation by chimeric core proteins

Core protein interact with pgRNA and polymerase for pgRNA incapsidation (Nassal and Schaller, 1996). And C-terminal region of core protein presumably binds to pgRNA during encapsidation (Nassal, 1992). To investigate whether this different sequence affects HBV pgRNA encapsidation, HBV nucleic acids within core
particles was examined first. Various constructs transfected cells were lysed and core particles were precipitated with PEG. Core particles were electrophoresed through a 1% agarose gel and transferred to nylon membrane. HBV nucleic acids in core particles were estimated by hybridization with a $^{32}$P-labeled HBV DNA probe after disruption of particles in situ (Gazina et al., 2000). From one of assembly competent chimeric core particle by HD 221-262 core proteins, HBV nucleic acids were detected meaning encapsidated HBV pgRNA and/or HBV DNA while chimeric core particle by HD 192-220, another assembly competent chimera, was unable to encapsidate pgRNA. HBV nucleic acids were detected from wt core particles produced by HBV wt-transfected cells and HBV core protein co-transfected cells (Fig. 4A). It should be noted that this assay could not discriminate whether detected HBV nucleic acids are encapsidated pgRNA or synthesized DNA. For this reason, RPA was conducted to detect encapsidated pgRNA from transfected HuH7 cells. Encapsidated pgRNA was detected from HD 221-262 chimera co-transfected cells like HBV wt core particles and this result is same with the previous assay (Fig. 4B). Encapsidated pgRNA level was higher from HBV wt transfected cells in which core proteins were provided in cis, than that from cells transfected with HBV core or HD 221-262 chimera in which core protein is were provided in trans. Therefore the different encapsidated pgRNA level may account for different supply of core proteins (Weizsacker et al., 2002).
Fig. 3. Chimeric core particle formation. Western blot analysis was performed to detect core particles from a native agarose gel electrophoresis. Isolated core particles were transferred to PVDF membranes, and incubated with anti rabbit HBC antibody. HRP-conjugated secondary antibody and ECL were used to visualize core particles. As positive and negative controls, HBV wt and C deficient mutant were employed.
Fig. 4. HBV pgRNA encapsidation by chimeric core particles. (A) HBV nucleic acids detection from disrupted core particles in situ. Isolated core particles were electrophoresed through a 1 % agarose gel and transferred to nylon membrane. HBV nucleic acids in core particles were estimated by hybridization with a $^{32}$P-labeled HBV DNA probe after the disruption of particles in situ. (B) RNase Protection Assay to detect encapsidated pgRNA. *In vitro* transcribed radiolabeled antisense RNA probe (446 nts) was hybridized overnight at 50°C with pgRNA from isolated core particles. Protected pgRNA (369 nts) following RNase digestion was run on a 5 % polyacrylamide-8M urea gel and visualized by autoradiography.
E. HBV DNA replication in chimeric core particles

Previous studies using series of C-terminally truncated HBV core proteins suggest that the core protein takes an active part in HBV replication via its C-terminal domain and that the arginine-rich domain has influence on full size DNA synthesis (Nassal, 1992; Pogam et al., 2005). Chimeric proteins that encapsidate pgRNA has full sized C-terminal region but has substitutions in several sequences (Fig. 1A). To further investigate that chimeric core protein can support HBV DNA replication, cytoplasmic core particles were isolated from HBV wt transfected or C deficient and chimeric core protein co-transfected HuH7 cells. Also, isolated core particles were tested to show that sequence substitution could influence the full-length DNA synthesis. Following extraction of HBV DNA from isolated core particles, Southern blot analysis was performed to detect replicating HBV DNA. Single-stranded, double-stranded, and relaxed circular HBV DNAs were observed from core particles from HBV wt transfected cells and with reduced intensity from cells cotransfected with C deficient mutant and HBV core protein (Fig. 5). Shorter DNA than single-stranded DNA genome of HBV was detected from HD 221-262 chimeric core particles with weak intensity. With shorter exposure to clearly see the single-stranded DNA genome of HBV wt, shorter size than genome length was evident (Fig. 5, lower panel). This result represents that sequence substitution at C-terminal region of core protein can support HBV DNA synthesis, but not the full sized HBV DNA genome.
**Fig. 5. HBV DNA replication in chimeric core particle.** Southern blot analysis was performed to detect HBV DNA synthesis in chimeric core particle. HBV DNA was extracted from isolated core particles, separated, transferred to nylon membranes, hybridized with a random-primed $^{32}$P-labeled HBV specific probe, and subjected to autoradiography. Single-, double-stranded, and partially double-stranded relaxed circular forms of HBV DNA are marked as SS, DS, and RC, respectively. Lower panel shows the shorter exposure of upper panel.
**Fig. 6. Summary of results.** The replication of C deficient mutant through trans-complementation by core protein and chimeric core proteins are summarized.

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IV. DISCUSSION

The C-terminal of HBV core protein contains protamine-like region that is rich in arginine residue, and is known as nucleic acid binding domain. C-terminal portion of core protein is dispensable for capsid particle assembly. However many researches with series of C-terminally truncated core protein mutants showed the importance of C-terminal portion of hepadnavirus core protein in pgRNA encapsidation and DNA replication. In this study, chimeric core protein expression plasmids were constructed by substituting the C terminal region of HBV core protein to DHBV to identify critical residues or motif at C terminal region of core protein for HBV replication.

Trans-complementation of C deficient mutant by core protein and chimeric core proteins are summarized in Fig. 6. Among three chimeras, HD 192-262 was unable to assemble core particle and HD 192-220 can assemble into core particle but cannot encapsidate HBV pgRNA nor synthesize HBV DNA. HD 221-262 can trans-complement HBV core protein for both HBV pgRNA encapsidation and HBV DNA synthesis but only smaller DNA than HBV genomic DNA was detected.

HD 221-262 chimeric core protein has sequence substitutions at C-terminal domain of HBV core protein without insertion or deletion. Through alignment of substituted region of HD 221-262, C-terminal domain of HBV and DHBV core protein has higher homology with 45.2 % homology sequences, while 26.7 % of homology with whole core protein sequences (Fig. 1A). From trans-complementation
results of HD 221-262 and sequence alignment, it indicates that homologous amino acids at C-terminal domain of core protein, displayed as yellow, is crucial for HBV pgRNA encapsidation. From previous studies, C-terminally deleted core protein with 1-164 amino acids in ayw subtype could encapsidate pgRNA (Nassal, 1992; Pogam et al., 2005). This result showed that amino acid from 165 to 183 is dispensable for encapsidation in ayw subtype. This could be applied to our results (adw subtype) in which corresponding amino acids from 166 to 185 is dispensable for encapsidation. For encapsidation, crucial amino acids may be Thr 146 to Val 149, Arg 152, Arg 154, Ser 157, Arg 161, Arg 162, Arg 163, and Pro 165.

It was suggested that amino acids from 165 to 173 residues of core protein (amino acid number of ayw subtype) may be important for full-length of HBV DNA synthesis while 174 to 183 residues is dispensable (Nassal, 1992). Recently it had been shown that Arg 172 and 173 (ayw subtype), the positively charged of amino acid, are required for full-length DNA synthesis (Pogam et al., 2005). HD 221-262 chimeric core protein trans-complement C deficient mutant for pgRNA encapsidation and DNA synthesis, while it cannot trans-complement full-length HBV DNA synthesis. This indicates that amino acid differences between HBV and DHBV sequences of HD 221-262 is crucial for synthesis of full-length HBV DNA except from 178 to 185 of adw subtype. Taken together, it can be proposed that crucial residues for full-length of HBV DNA may be Arg 168, Arg 169, Gln 171, Ser 172, and Arg 175.

Recent studies reported that core particles assembled from core protein with
truncation or insertion were unstable (Kock et al., 2004; Kock et al., 1998; Pogam et al., 2005). DHBV was defective in DNA replication when DHBV core protein had insertions for up to 25 residues at N-terminus, possibly due to earlier nucleocapsid destabilization (Kock et al., 1998). This destabilization by insertional mutation might explain the failure of trans-complementation by HD 192-220 chimeric core protein. HD 192-220 chimeric core protein has insertion of 29 residues between amino acid 145 and 146 of HBV core protein without sequence substitution. HD 192-220 cannot rescue HBV pgRNA encapsidation and DNA synthesis except core particle assembly. It is still possible to speculate that 29 residues insertion causes destabilization of core particle, thus encapsidated pgRNA cannot be protected from nuclease treatment for core particle isolation procedure. HD 192-262 chimera was unable to assemble into core particle even though it has same length of inserted residues like HD 192-220. Since HD 192-262 has more extensive alterations, C-terminal substitution and 29 residues insertion, these alterations may cause the destabilization of core particles (cannot maintain particle structure) or the incompetency of core particle assembly.

It had been reported that spliced RNA of hepadnavirus was encapsidated and DNA was synthesized from this spliced pgRNA inside core particles by C-terminally truncated core protein (Kock et al., 2004; Pogam et al., 2005). It is possible that short DNA detected from HD 221-262 chimeric core particle may be synthesized from spliced RNA. This should be investigated in detail.
V. CONCLUSION

In this study, to identify the nucleic acid binding domain for HBV replication, chimeric core proteins of HBV were constructed by substituting HBV core protein C terminal region to corresponding DHBV sequences. DHBV core proteins that consist of 262 amino acids can form a three-dimensional nucleocapsids structure similar to that of HBV. Chimeric core proteins were designed to contain various lengths of corresponding DHBV sequences, while N-terminal sequences of HBV core protein were unchanged. With these chimeric core proteins, HBV pgRNA encapsidation and HBV DNA replication were examined. This chimeric core protein study showed that chimeric core protein can trans-complement HBV core proteins for C deficient mutant. This study also shows that the 45.2% of amino acid sequence identity at core protein C-terminal region for nucleic acid binding domain is sufficient to encapsidate HBV pgRNA and synthesize HBV DNA.
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- 국문요약 -

키메라 캡시드 단백질로부터 형성된 캡시드 입자의
B형 간염 바이러스 복제능력

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B형 간염 바이러스의 뉴클레오패시드를 형성하기 위해서는 캡시드 단백질, DNA 중합효소, pgRNA의 상호작용이 필요하다. 이 중 캡시드 단백질의 N-말단은 캡시드 입자의 조립에 관여하며 그 자체만으로도 캡시드 조립이 가능하다. 또한 C-말단은 캡시드 입자의 조립에는 관여하지 않지만 핵산 결합 부위로서 pgRNA를 감싸거나 DNA를 복제하는 과정에 관여한다. 그러나 C-말단의 이러한 작용에 중요한 역할을 하는 아미노산이나 도메인은 아직까지 불분명하다. 따라서 이 연구에서는 B형 간염 바이러스 캡시드 단백질의 C-말단을 오리 B형 간염 바이러스 캡시드 단백질로 치환한 키메라 캡시드 단백질을 이용하여 핵산 결합 부위의 중요한 아미노산이나 도메인을 밝히고자 하였다. 키메라 캡시드 단백질은 B형 간염 바이러스 캡시드의 N-말단에는 변화 없이 C-말단을 다양한 길이의 상응하는 오리 B형 간염바이러스 캡시드 단백질의 C-말단으로 치환하도록 제작하였다. 각각의 키메라 캡시드 단백질을 B형 간염 바이러스의
캡시드 결손 돌연변이주와 함께 HuH7 세포에 발현시켰다. 이를 통해 pgRNA와 캡시드 단백질을 제외한 구조 단백질은 캡시드 결손 돌연변이주로부터 제공되고 캡시드 단백질은 키메라 단백질이 제공하도록 하였다. 일부 키메라 캡시드 단백질이 캡시드 입자를 형성할 수 있었고 그 중에 하나는 pgRNA를 감싸거나 DNA를 합성할 수 있었다. 이 키메라 캡시드 단백질을 분석한 결과 오리 B형 간염바이러스의 캡시드로 치환한 C-말단의 아미노산 서열 중 45.2 %가 상동 관계였다. 그러므로 이 결과는 상동관계에 있는 45.2 %의 아미노산이 pgRNA를 감싸고 HBV DNA를 복제하는데 중요한 부분이라는 것을 보여준다.

핵심어 : 키메라 캡시드 단백질, B형 간염 바이러스의 복제
Hepadnaviruses are small, enveloped DNA viruses that replicate with narrow host range and a relative tropism for the liver (Wands and Blum, 1991). And hepadnaviruses have unique replication strategy that involves reverse transcription of a pgRNA (Seeger and Hu, 1986). HBV, a prototype of hepadnavirus, has a partially duplex relaxed circular DNA genome that contains overlapping reading frames encoding the core protein, polymerase, surface proteins, and X protein. Hepadnavirus polymerase, a multifunctional protein, plays roles in DNA synthesis and encapsidation of pgRNA (Bartenschlager et al., 1990; Hirsch et al., 1990), and acts as a primer for minus-strand DNA synthesis (Wang and Seeger, 1992; Weber et al., 1994; Zoulim and Seeger, 1994). The polymerase consists of four domains, terminal protein (TP), spacer, reverse transcriptase (RT), and RNase H. The TP domain acts as a protein primer for HBV minus-strand DNA synthesis by covalently linked with first nucleotide of minus-strand DNA. The spacer region connects the TP and RT/RNase H domains. This region is highly divergent between hepadnaviruses and appears to be non-essential, since substitution, deletion and insertional mutations are tolerated. RT and RNase H regions of HBV polymerase display homology with RT of retrovirus, which has an active and conserved YMDD motif for RNA-dependent DNA polymerase and a conserved RNase H motif.

As mentioned above, HBV polymerase recognizes other viral components for
encapsidation and acts as a protein primer beside reverse transcriptase activity. After translation, HBV polymerase recognizes encapsidation signal, epsilon (ε) sequence, of pgRNA and forms RNP complex through ε sequence of pgRNA for encapsidation. This RNP complex is recognized by core protein then packaged into core particle. Inside core particle, reverse transcription occurs from pgRNA. At this point, polymerase is used as a primer for minus-strand HBV DNA synthesis. Covalent linkage between the hydroxyl group of Tyr in the TP domain of HBV polymerase and first nucleotide is formed by phosphodiester bonds (Lanford et al., 1997; Weber et al., 1994; Zoulim and Seeger, 1994). After this priming reaction, three or two nucleotides are subsequently copied from the bulge region of ε to make the nascent minus-strand DNA. Following the synthesis of nascent minus-strand DNA, the oligomer covalently linked to the polymerase translocates from ε to direct repeat (DR) 1 at the 3'-end of pgRNA, leading to the elongation of minus-strand DNA (Tavis and Ganem, 1995; Tavis et al., 1994).

Previous study had shown that the priming deficient mutant polymerase, which has substitution of Tyr to Phe, can synthesize short oligomer, the nascent minus-strand DNA without priming reaction (Kim et al., 2004). Since Phe has similar structure with Tyr except hydroxyl group, there should be no structural alteration of polymerase even though phosphodiester bond between TP domain and first nucleotide cannot form. Since that report showed that HBV polymerase can initiate minus-strand DNA synthesis without a primer, that raised an important insight that
HBV DNA polymerase may have property like RNA polymerase, such as the primer independent initiation.

Recently, several studies have indicated that the identity of each classes of polymerase have been blurred according to template specificity and nucleotide substrate selectivity (Joyce, 1997). It has been reported that single amino acid mutation can change property of DNA polymerase to reverse transcriptase (Beck et al., 2002; Cases-Gonzalez et al., 2000; Gao et al., 1997).

In this study, RNA polymerase activity of HBV DNA polymerase, was explored by testing the NTP incorporation capacities. NTP incorporation was examined with mutant polymerase which has single amino acid substitution from Phe 436, the bulky amino acid, to smaller amino acids in dNTP binding cleft. As a result, HBV polymerase with substitution in dNTP binding cleft can incorporate NTPs. This suggests that HBV DNA polymerase obtain one of the properties of RNA polymerase by single amino acid change. Together with primer independent initiation of DNA synthesis (Kim et al., 2004) and the NTP incorporation capacity by single amino acid substitution, it suggests that HBV DNA polymerase could acquire RNA polymerase property with minor modification.
II. MATERIALS AND METHODS

A. DNA construction

HBV subtype adw R9 plasmid construct, pPB, was used as a wild-type (wt) HBV construct (Kim et al., 2004). Phe 436, the bulky amino acid in dNTP binding cleft of HBV DNA polymerase was changed to Gly using a fusion PCR technique. Forward mutagenic primer HBV 79 (5’-GAATATGATACCCCGCGACAGACATCCAG-3’) targeting nts 415 to 375 and reverse primer HBV 3 (5’-CCACCTCTAAAGAGACAGTC-3’) targeting nts 3183 to 3201 was used for PCR reaction. In another PCR reaction, reverse mutagenic primer 78 (5’-GTCTGCGGCGGTTATCATATCTTCTTCTCATC-3’) targeting nts 383 to 414 and forward primer HBV 8 (5’-CACGATCGGTACAGACTTG-3’) targeting nts 783 to 764 was used. Resulting PCR products of 443 bp and 369 bp fragments were combined by fusion PCR. Fusion PCR product was digested with XcmI and EcoRI then cloned into corresponding enzyme restriction site of HBV wt plasmid, pPB (Kim et al., 2004), yielding RT F436G.

To construct RT F436V, which is Phe 436 of HBV polymerase was changed to Val, same cloning procedure, as above, was used except the mutagenic primers. Reverse primer HBV 80 (5’-GTCTGCGGCGGTTATCATATCTTCTTCTCATC-3’) and forward primer HBV 81 (5’-GAATATGATACCCCGCGACAGACACAT
CCAG) were mutagenic primer set for RT F436V construction.

RT reaction-deficient RT YMHA construct, the conserved YMDD motif was modified to YMHA, was used as negative control (Kim et al., 2004).

B. Cell culture and transfection

HuH7 hepatoma cell lines were used for the transfection of HBV wt and mutant clones. HuH7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with penicillin and streptomycin, and 10% fetal bovine serum. HBV wt or mutants plasmids (8 µg) were transfected into HuH7 cells on a 10 cm plate using Lipofectamine (Invitrogen™, Gaithersburh, MD, U.S.A.), according to the manufacturer’s instructions.

C. Isolation of core particles

Three days after transfection, HuH7 cells were lysed in 1 ml TNE (10 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1 mM EDTA) containing 1% Nonidet P-40. The clarified lysate was adjusted with 10 mM MgCl₂ and 8 mM CaCl₂ solution, and incubated overnight at 37°C with 20 U DNase I (SIGMA, St. Louis, MO) and 60 U micrococcal nuclease (Calbiochem, San Diego, CA). Cytoplasmic core particles were precipitated with 6.5% polyethylene glycol.
D. RNase protection analysis (RPA)

To analyze encapsidated pgRNA, core particles were isolated as described above. PgRNA from core particles was extracted following proteinase K (100 µg/ml) and DNase I (20 U) treatment. To prepare riboprobe for RPA, part of the HBV sequence (nts 1805 to 2187) was cloned into pGEM3Zf(+) vector, generating pRPAFD. From this construct, 446 nts of radiolabeled anti-sense probe were synthesized in vitro using SP6 RNA polymerase with α-32P-UTP (specific activity, 800 Ci/mmol). The RPA procedure was performed using the manufacturer’s protocol (RPA II™, Ambion). Protected pgRNA was 369 nts in length.

E. Northern and Southern Blotting

Total RNA (10 µg) was denatured and electrophoresed on a 1% agarose gel containing formaldehyde, and blotted onto a nylon membrane. RNA on the membrane was hybridized to a 32P-labeled random-primed probe specific for the HBV sequence. To analyze HBV DNA synthesis by Southern blotting, core DNA was extracted, separated by agarose gel electrophoresis, and hybridized to a 32P-labeled random-primed probe specific for the HBV sequence.
F. Core particle Western blotting

Isolated core particles were electrophoresed on a 1% native agarose gel (Koschel et al., 2000). Core particles were transferred to polyvinylidene fluoride (PVDF) membranes, as described for Northern blot analysis. Immunoblotting was performed using an anti-HBc antibody (DAKO, Glostrup, Denmark). Horseradish peroxidase-conjugated anti-rabbit secondary antibody and ECL (Enhanced Chemical Luminescence) were employed to visualize HBV core particles.

G. Endogenous polymerase assay (EPA)

Isolated core particles were incubated at 37°C overnight with EPA reaction buffer (50 mM Tris-HCl [pH 7.5], 75 mM NH₄Cl, 1 mM EDTA, 25 mM MgCl₂, 0.1% β-mercaptoethanol, 0.5% Nonidet P-40) supplemented with 0.5 mM each of dCTP, dGTP, and TTP and 10 μCi α-³²P-dATP or α-³²P-ATP (specific activity, 3000 Ci/mmol). ³²P-labeled reaction mixtures were electrophoresed on a 1% agarose gel and subjected to autoradiography (Kim et al., 2004).
III. RESULTS

A. Construction of HBV DNA polymerase mutant

DNA polymerases, including reverse transcriptase, have a bulky residues at
motif A either an aromatic amino acid or a Glu (Joyce, 1997). This residue in dNTP
binding cleft is crucial for the selection of dNTPs instead of NTPs that act as a steric
gate to prevent the larger NTPs in the pocket. Mutational studies reported that crucial
bulky residue for dNTP selection is the Phe 155 of MMLV reverse transcriptase, the
Tyr 115 of HIV-1 reverse transcriptase and the Phe 451 of DHBV reverse
transcriptase and the substitution of this bulky residue to smaller amino acid residue
confer NTPs incorporation capacity on reverse transcriptase (Beck et al., 2002;
Cases-Gonzalez et al., 2000; Gao et al., 1997). Through amino acid sequence
alignment, corresponding amino acid of HBV DNA polymerase was found to be the
Phe 436 in RT domain of polymerase (Fig. 1).

To investigate the role of Phe 436 of HBV DNA polymerase for dNTP
selectivity, Phe 436 was replaced to smaller amino acids, Gly or Val. HBV wt with
subtype adw R9 was constructed (Blum et al., 1991) in which transcription of
pgRNA is controlled by a CMV IE promoter (Kim et al., 2004). Next, Phe 436 of
HBV DNA polymerase was changed to smaller amino acids, Gly or Val, generating
two polymerase mutants RT F436G and RT F436V. Additionally, RT reaction-
deficient RT YMHA mutants were constructed in which the conserved YMDD reverse transcriptase motif was modified to YMHA (Fig. 2).
Fig. 1. Reverse transcriptase alignment of HBV polymerase with DHBV polymerase, HIV-1, and MoMLV. In diagram of polymerase, individual domain of polymerase is presented as boxes. And dark boxes indicate relative positions of the motif 1 and 2, and A to E, the conserved motifs. Sequence alignment of motif A is shown below. Conserved bulky amino acid is highlighted as an arrowhead.
Fig. 2. Schematic diagram of HBV wt and mutants constructs used for transient expression. HBV sequences are depicted as thick lines and the ε sequences at 5’- and 3’-ends are marked. The ORF of C, P, S and X genes are presented as open boxes. Each domain (TP, spacer, RT and RNase H) of the polymerase is indicated. The CMV promoter presented as an open arrow. Mutant constructs that have substitution of Phe 436 to Gly or Val of RT, and RT YMDD to YMHA of wt HBV adw R9 background, are designated RT F436G, RT F436V, and RT YMHA, respectively. The positions of polymerase point mutations are indicated as closed arrowheads.
B. HBV RNA transcription and core particle formation

HBV RNA expression was analyzed from wt-or polymerase mutants-transfected HuH7 cells. Total RNA was analyzed by Northern blot analysis. Upon transfection, pgRNA was expressed by the action of the CMV promoter. Subgenomic RNA sequences were synthesized under control of their authentic promoters. The 3.5 kb pgRNA, and 2.1 and 2.4 kb mRNA of surface proteins were detected from wt or mutants-transfected cells (Fig. 3A). There were no significant differences in the RNA expression levels between wt and mutants-transfected cells (Fig. 3A).

Core particles formation was examined by native agarose gel electrophoresis and Western blotting, using the anti-HBc antibody (Koschel et al., 2000). Core particles were detected from wt or polymerase mutants-transfected HuH7 cells (Fig. 3B). Core particle formations by all of polymerase mutants were similar with HBV wt.
Fig. 3. HBV RNA transcription and core particle formation. (A) Northern blot analysis to detect HBV mRNA expression. Total RNA was extracted and separated by 1% formaldehyde gel electrophoresis, transferred to nylon membranes, hybridized with random-primed $^{32}$P-labeled HBV specific probes, and subjected to autoradiography. pgRNA (3.5 kb) and 2.1 and 2.4 kb mRNA for surface protein(S) are indicated. (B) Western blot analysis to detect HBV core particles from a native agarose gel. Isolated core particles were transferred to PVDF membranes and incubated with rabbit HBc antibody. HRP-conjugated secondary antibody and ECL were used to visualize proteins. HBV wt and all of the mutants can form core particles.
C. HBV pgRNA encapsidation

HBV pgRNA encapsidation by series of polymerase mutants was investigated. RPA was performed to detect encapsidated pgRNA in core particles of wt and mutants-transfected HuH7 cells. To encapsidate pgRNA, HBV DNA polymerase recognizes encapsidation signal (ε) of pgRNA and forms RNP complex. RNP complex is recognized and encapsidated by dimers of core proteins to form core particles. It was speculated that there should be no significant alteration in tertiary structure of RT F436G and RT F436V polymerase mutants except dNTP binding cleft compared to that of HBV DNA polymerase. Thus RT mutations should not affect pgRNA encapsidation. As expected, RT F436G, RT F436V and RT activity-deficient RT YMHA mutants encapsidated pgRNA as well as HBV wt (Fig. 4). This data showed that mutation in dNTP binding cleft of polymerase do not change the tertiary structure of polymerase except dNTP binding cleft and thus pgRNA can be encapsidated by these mutant polymerases.
**Fig. 4. HBV pgRNA encapsidation.** RNase Protection Assay was performed to detect HBV encapsidated pgRNA. *In vitro* transcribed radiolabeled antisense RNA probe (446 nts) was hybridized overnight at 50°C with extracted pgRNA from isolated core particles. Protected pgRNA (369 nts) following RNase digestion was run on a 5% polyacrylamide-8M urea gel and visualized by autoradiography.
D. HBV DNA replication by polymerase mutants

HBV DNA synthesis may be affected by the mutation of HBV polymerase in dNTP binding cleft. To investigate HBV DNA synthesis by dNTP binding cleft mutation, HBV DNA synthesis in core particle was analyzed. From isolated core particles, HBV DNA was purified and then southern blot analysis was performed. HBV wt showed single-stranded, double-stranded, and partially double-stranded relaxed circular HBV DNA but two dNTP binding cleft mutants, RT F436G and RT F436V, and RT-deficient mutant did not show any HBV DNA (Fig. 5). RT-deficient RT YMHA mutant did not support HBV DNA synthesis since conserved active reverse transcriptase motif of YMDD was changed to YMHA in this mutant (Chang et al., 1990; Hirsch et al., 1990). It has been speculated that HBV DNA polymerase with enlarged dNTP binding cleft by amino acid mutation can incorporate dNTPs with the reduced preference for dNTP than wt. However, southern blot analysis showed that dNTP binding cleft mutant polymerases were unable to synthesize HBV DNA. Two possibilities can be suggested: first, these supposedly dNTP binding cleft mutant polymerase cannot incorporate dNTPs. Second, it is possible that these dNTP binding cleft mutant polymerase can incorporate dNTP, thus synthesize short oligomer of DNA, but was lost during HBV DNA purification from core particles. These possibilities were tested in the following section through new assay system (Kim et al., 2004).
Fig. 5. HBV DNA synthesis by mutant polymerase. Southern blot analysis was performed to detect HBV DNA replication. HBV DNA was extracted from isolated core particles, separated, transferred to nylon membranes, hybridized with a random-primed $^{32}$P-labeled HBV specific probe, and subjected to autoradiography. Single-, double-stranded, and partially double-stranded relaxed circular forms of HBV DNA are marked as ssDNA, dsDNA, and RC, respectively.
E. NTP incorporation by dNTP binding cleft mutant polymerase

Substitutions of Phe 436, the bulky amino acid in dNTP binding cleft of HBV polymerase, to Gly or Val, the smaller amino acids, may enlarge dNTP binding cleft. With these mutants, NTPs incorporation capacity of HBV DNA polymerase was investigated. To detect short oligomer synthesized by mutant polymerases, endogenous polymerase assay (EPA) was performed by incubating the isolated wt and mutant core particles with $^{32}$P-labeled nucleotide (Kim et al., 2004). At first, dNTP incorporations of core particles of HBV wt and polymerase mutant were tested. Isolated intracellular core particles were incubated with $^{32}$P-labeled dATP and cold dNTPs. During this reaction, active polymerase in core particle can incorporates labeled or cold dNTPs into growing DNA. Consequently, radioactively labeled DNA or oligomers in core particles should be detected by autoradiography following the native agarose gel electrophoresis of core particles, but not by inactive polymerase in core particles with free radioactive $^{32}$P-dATP. After EPA reaction, reaction mixtures were electrophoresed on 1% native agarose gels and subjected to autoradiography. As expected, wt core particles were labeled with strong intensity (Fig 6A). RT F436G and RT F436V polymerase mutant core particles were labeled with weak intensity than wt. RT F436V core particle show stronger signal than RT F436G. RT YMHA core particles were not labeled because reverse transcriptase activity was abolished by mutations. This result indicates that polymerase which has dNTP binding cleft mutation can incorporate dNTPs and synthesize short DNA oligomer
inside of core particles.

Then NTP incorporations of core particles were examined in HBV wt and polymerase mutant. Isolated core particle was subjected in modified EPA with $^{32}$P-labeled ATP and cold dNTPs. If the polymerase in core particle can incorporate $^{32}$P-ATP, core particle should be radioactively labeled, but in the opposite case, no core particle should be labeled and detected by autoradiography. Only RT F436G core particle was detected (Fig. 6B). This result indicates that HBV polymerase with bulky amino acid F436 substitution to smaller amino acid G, can incorporate ATP and synthesize chimeric RNA-DNA oligomer that consists of dNTP and NTP.
Fig. 6. Endogenous Polymerase Assay (EPA). (A) HBV DNA synthesis was examined by EPA. Isolated core particles were incubated with EPA reaction buffer supplemented with 0.5 mM each of dCTP, dGTP, TTP and 10 µCi α-32P-dATP (3000Ci/mmol) at 37 °C for overnight. (B) To investigate NTP incorporations, isolated core particles were incubated with EPA reaction buffer supplemented with 0.5 mM each of dCTP, dGTP, TTP and 10 µCi α-32P-ATP (3000Ci/mmol), instead of α-32P-dATP, at 37 °C for overnight. Reaction mixtures were electrophoresed on 1% agarose gel and subjected to autoradiography.
IV. DISCUSSION

Hepadnavirus DNA polymerases act as a protein primer for minus-strand DNA synthesis. Phosphodiester bonds between the hydroxyl group of Tyr in the TP domain and the first nucleotide involve in the priming of reverse transcription (Lanford et al., 1997; Weber et al., 1994; Zoulim and Seeger, 1994). Three or two nucleotides are subsequently copied from the bulge region of ε to make TGAA or GAA, the nascent minus-strand DNA. From the previous study, however, the priming deficient mutant polymerase, in which Tyr 65 of TP domain was changed to Phe, had ability to synthesize oligomer from the bulge region of ε as a template in the absence of covalent linkage between TP and first deoxynucleotide (Kim et al., 2004). This suggests the primer independent initiation of HBV DNA polymerase. Primer independent initiation mechanism is one of the properties of RNA polymerase.

Since HBV DNA polymerase might have RNA polymerase property, NTP incorporation capacity, the other property of RNA polymerase, was examined with HBV DNA polymerase in this study. NTP incorporation capacity was tested with the enlarged dNTP binding cleft mutant of HBV DNA polymerase. The dNTP binding cleft mutant can incorporate NTPs and synthesize chimeric oligomer consist of dNTPs and NTPs.

DNA polymerases, including reverse transcriptase, have a bulky residues at motif A, either an aromatic amino acid or a glutamate (Joyce, 1997). Mutational
studies of MMLV, HIV-1 and DHBV reverse transcriptase reported that this bulky residues, Phe 155, Tyr 115 and Phe 451, respectively, act as a steric gate to select dNTP and prevent the positioning of larger NTPs in the pocket (Beck et al., 2002; Cases-Gonzalez et al., 2000; Gao et al., 1997). This study demonstrates that Phe 436 of HBV polymerase is a bulky residue in dNTP binding cleft that acts as a steric gate for dNTP selection. Comparing the dNTP incorporation of RT F436G and RT F436V mutants, stronger dNTP signal was observed from RT F436V (Fig. 4A). Since Val is larger than Gly residue, dNTP binding cleft by Val of RT F436V is smaller than that of Gly of RT F436G and more similar than that of wt polymerase for dNTP binding. Thus dNTP incorporation by RT F436V mutant polymerase is more efficient than that by RT F436G polymerase. In contrast, NTP incorporation was observed only by RT F436G mutant polymerase. This result indicates that substitution of bulky amino acid, Phe 436, to smaller amino acid, Gly, is sufficient to enlarge dNTP binding cleft and to bind NTP. It is possible that RT F436V mutant polymerase might incorporate NTP but signal may be too weak. These different results according to the size of substituted amino acids are consistent with the previous report (Beck et al., 2002).

The possibilities that these mutations may indirectly affect the polymerase folding and other functions of polymerase could be excluded by the pgRNA encapsidation. PgRNA encapsidation by these polymerase mutants were similar with wt polymerase. This indicates that there is no alteration of polymerase folding and other functions such as the interaction with pgRNA and the encapsidation, except dNTP binding cleft.
Collecting data from Southern blot analysis and endogenous polymerase assay, HBV DNA synthesis is affected by mutation in dNTP binding cleft. These two dNTP binding cleft polymerase mutants are able to incorporate dNTP and synthesize short oligomer that is long enough not to pass through holes of core particle, but are unable to synthesize authentic HBV DNA.

Previous studies used in vitro assay system for the dNTP incorporation of reverse transcriptase and the bulky amino acid for dNTP selection. All of the experiments in this study were performed with authentic core particles from full HBV genome transfected cell culture system. Thus this study showed the NTP incorporation of mutant polymerase inside of intracellular core particle. Hence this is the first report that NTP incorporation can occur nearly authentic replicating condition.
V. CONCLUSION

In this study, RNA polymerase activity of HBV DNA polymerase, was explored by testing the NTP incorporation capacities. NTP incorporation was examined with mutant polymerase with single amino acid substitution from Phe 436, the bulky amino acid, to smaller amino acids in dNTP binding cleft. Since these mutant polymerases could encapsidate pgRNA, there should be no alterations on polymerase foldings, except in dNTP binding cleft. As a result, HBV polymerase with substitution in dNTP binding cleft can incorporate NTPs. This suggests that HBV DNA polymerase obtains one of the properties of RNA polymerase by single amino acid change. Together with primer independent initiation of DNA synthesis and the NTP incorporation capacity by single amino acid substitution, it suggests that HBV DNA polymerase could acquire RNA polymerase property with minor modification.
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- 국문요약 -

B형 간염 바이러스 DNA 중합효소의 역전사 기능

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B형 간염 바이러스 (HBV)의 DNA 중합효소는 RNA나 DNA를 주형으로 하여 DNA를 합성하는 기능을 갖고 있으며, 바이러스의 음성가닥 DNA를 만드는 과정에서 단백질 프라이머로서 작용하기도 한다. 이전의 연구에서 HBV DNA 중합효소의 시작 결손 변이주가 말단 단백질과 첫번째 디옥시올리고뉴클레오티드 사이의 공유결합 없이도 짧은 DNA 조각을 합성한다는 것을 밝힌 바 있다. 이 사실은 HBV DNA 중합효소가 프라이머 없이 DNA를 합성하는 RNA 중합효소의 특성을 갖고 있을 가능성이 있다는 것을 제시한다. 본 연구에서는 NTP 결합능력을 관찰함으로써 HBV DNA 중합효소에서 RNA 중합효소의 특성을 조사하였다. HBV DNA 중합효소의 dNTP 결합부위에 부피가 큰 아미노산을 부피가 작은 글라이신이나 발린으로 변이시킨 후, NTP 결합능력을 향상하는 자를 확인하였다. 그 결과 HBV DNA 중합효소의 dNTP 결합부위에 위치하는 부피가 큰 436번 패닐알라닌을 글라이신으로 변이시킨 중합효소 변이주가 HBV DNA를 합성할 수는 없었지만 NTP 결합능력이 있음을
확인하였다. 이 결과는 HBV DNA 중합효소의 dNTP 결합부위에 있는 436번 페닐알라닌이 dNTP 선택에 있어서 공간적 관문으로 작용한다는 사실을 의미한다. 본 연구의 결과는 단일 아미노산의 치환에 의하여 DNA 중합효소와 RNA 중합효소 특성의 경계가 불분명해질 수 있음을 나타낸다.

핵심어 : HBV DNA 중합효소, dNTP 결합부위, NTP 결합