

Expression of the Coxsackie-Adeno-Virus Receptor on Pancreatic β -Cells of Rat and Human OriginAsma Elshebani^{1,2*}, Anna-Karin Berg¹, Gun frisk¹¹Department of Women's and Children's Health, University Hospital, Uppsala, Sweden²Department of Infectious Diseases, Faculty of Public Health, Benghazi University, Benghazi, Libya**Original Research Article*****Corresponding author**

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Abstract: Type 1 diabetes mellitus is a disease that results from autoimmune destruction of the insulin-producing beta-cells in the pancreatic islets of Langerhans. This process is induced in genetically susceptible individuals by some, still not known, environmental factors. These environmental factors are believed to be enterovirus infections, which have a significant role in initiation and development of the disease. The first requirement for infection is the presence of a virus receptor on the host cell. The coxsackievirus and adenovirus receptor (CAR) is a common receptor for all the coxsackievirus B serotypes, which is a member of the enterovirus family. In this project my aim was to investigate the expression of human CAR mRNA in human pancreatic islet cells, and to investigate the expression of rat CAR mRNA in rat cell line (RINm5F) cultured as monolayer and free floating cell clusters (RCC). The investigation was done by two methods, one that analyzed presence of the CAR gene and one that analyzed the presence of the CAR protein. Results show that the receptor protein CAR was expressed in pancreatic beta-cells of rat and human.

Keywords: CAR receptor, beta-cell, Coxsackievirus, Pancreatic islet cell.

INTRODUCTION

The Coxsackie-Adeno-virus receptor (CAR) is a 46kDa-membrane glycoprotein (~365 amino acids) belonging to the immunoglobulin super family and containing two extracellular domains (D1 and D2), a membrane-spanning helical domain, and an intracellular domain.

The cytoplasmic domain D1 (V-like module) is necessary and sufficient for adenovirus binding [1] and the transmembrane domain D2 (C2-like module) it has been shown to possess all of the necessary characteristics for coxsackievirus B (CVB) attachment and entry when anchored to the membrane by glycosylphosphatidyl inositol [2, 3]. The protein of is bound to the outer membrane of many tissue types, including those from the heart. However, it was shown recently by using RT-PCR, that CAR exists as three isoforms, and that lack of the transmembrane domain, is the result of alternative RNA splicing. These alternatively spliced CAR proteins are released from transfected Hela cells, confirming that they are soluble proteins [4].

Therefore, it was identified as a common cellular receptor for both viruses [5-8]. Its biological and pathogenic function is still unclear, although it may be a component of the tight junction between polarized epithelial cells [9], and it has been suggested to play a role as an adhesion molecule during brain development [10].

CAR expression, which is relevant for both viral pathogenesis and gene therapy with

CAR-dependent vectors, is highly variable in different tissues in different species. For example CAR is expressed in the exocrine pancreas [11] and in the heart and brain of mice and humans [8]. These tissues are often affected by CVB infection. It is also expressed in mouse liver, lung and kidney [8]. In addition, the CAR expression in human myocardial is highly variable [12]. In mice, CAR expression has been reported to be high in acinar cells of the pancreas but barely detectable in the pancreatic islets [11]. Intriguingly, CAR often seems to be induced in regenerating cells. CAR is not usually detectable in normal mature rat hearts and brain [6], it is induced in the regenerating cells in an adult rat model of autoimmune-mediated myocarditis, and in the pancreas of adult mice [13, 11, 8].

Enteroviruses, in particular CBV, have been associated with type 1 diabetes (T1D)[14-19]. All serotypes of CVB including the E₂ strain of coxsackie virus B4, which is a diabetogenic strain in mice, have

been shown to use CAR as a receptor for attachment as well as for entry [20, 21].

CVB is believed to be able to infect and kill the insulin-producing cells (β -cells) directly [22-26]. This underlines the importance of studying expression of virus receptor in these cells, since the first requirement for infection is the presence of a virus receptor on the host cell. In many cases several host cell proteins are required for attachment, internalisation, and uncoating of the virus, which means that tropism and pathogenicity might depend on the presence of the appropriate host cell proteins [27].

The aim of this project was to study if CAR mRNA could be detected in pancreatic β -cells, of both rat and human origin, and if CAR protein was expressed on the cell surface.

MATERIALS AND METHODS

Cell Culture

The RINm5F cells are from a rat insulinoma, meaning that the β -cells have become cancer cells and formed a tumour; from that tumour the cell line was derived.

RINm5F cell line (kindly provided by Prof. ClaesHellerström, Dept. Medical Cell Biology, Uppsala University) were cultured as monolayer at 37°C 5% CO₂, in Eagle's Minimum Essential Medium (EMEM) (SVA, Uppsala, Sweden) supplemented with 10% newborn bovine serum (defined supplemented cat no. 3H30072.03,

Hyclone, Logan, UT). After a few passages of some of the RINm5F cells cultures mushroom-like cell clusters began to grow in the confluent monolayer. These gradually formed islet-like cell clusters that detached with increased growth, leaving a crater in the confluent cell layer see Figure 1. The RIN cell clusters (RCC) were then subsequently maintained free-floating in culture.

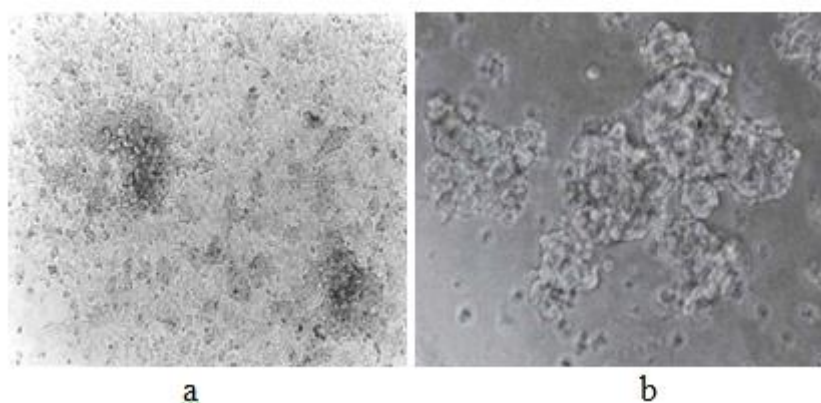


Fig-1: Formation of RINm5F cell clusters (RCC). The RCC were detached from the RINm5F monolayer (a) cells cultured in EMEM supplemented with 10% new-born bovine serum and subsequently kept as free floating RCC (b)

The human pancreatic islets (kindly provided by Prof. OlleKorsgren, Dept. Clinical Immunology, and Uppsala University) were cultured as described by Johansson *et al.* [28]. Briefly, the islets were cultured in untreated petri dishes Sterilin (Tamro Med. Lab. AB) and kept at 37°C in an atmosphere of 5 % CO₂ in humidified air in culture medium, RPMI 1640 (Gibco-BRL, SVA) supplemented with 10% new-born bovine serum (defined supplemented cat no. 3H30072.03, Hyclone, Logan, UT).

RNA preparation

RNA was extracted from RINm5F cells cultured as RCC, and from human pancreatic islet cells

homogenized (50-100 islet in 100 μ l distilled water) and frozen at -70 °C before extraction. Total RNA was isolated from the cells by the use of Rneasey Mini Kit (Qiagen, QiagenGmbH, Germany). The extracted RNA was stored at -70 °C. RNA was also extracted from the RINm5F cells cultured as monolayer in 12-well plates or cell culture flasks after they were harvested with a cell scraper, washed three times with 500 μ l PBS (phosphate-buffer saline) and frozen after addition of 50 μ l RNAlater buffer at -70C. RNA was isolated from the cells also using the Rneasey Mini Kit (Qiagen, QiagenGmbH, and Germany). Extracted RNA was stored at -70 °C.

Primers

Table-1: Primers used for amplification

Name	sequence	amplicon length (bp)
rCARs	CTCCTGGGGTTGCAAATAGA	181
rCARa	GGCATTCTGGGAATCTGA	181
rat β -Actin s	CACGGCATTGTAACCAACTG	581
rat β -Actin a	GGACCCCAGGGATGAATGAT	581
hCARs	GCAGGAGCCATTATAGGAACCTTG	195
hCARa	GGACCCCAGGGATGAATGAT	195
human β -Actin s	ACTGGAACGGTGAAGGTGAC	350
human β -Actin a	CTCAAGTTGGGGGACAAAAA	350

s(sense) a (antisense).

- The primers were selected with the use of the primer3 program.

Reverse transcription (RT)

The RNA was reverse transcribed using Sensiscript Reverse Transcriptase Kit for first-strand cDNA synthesis (Qiagen, QiagenGmbH, Germany), in a volume of 20 μ l. A master mix containing 10x buffer RT 2 μ l, dNTP mix 2 μ l, Oligo-dT primer 2 μ l, Rnase inhibitor 1 μ l, Sensiscript RT 1 μ l, Rnase-free water 11 μ l was mixed and vortexed for 5 sec. then centrifuged briefly and stored on ice. The template RNA (1 μ l) was added, and the sample vortexed and centrifuged as before, followed by incubation for 60 min at 37°C. The reaction was then heated to 93°C for 5 min followed by rapid cooling on ice.

Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) was performed using the Taq PCR Master Mix Kit (Qiagen, QiagenGmbH, and Germany). 1 μ l of template cDNA was amplified in a volume of 50 μ l containing 25 μ l Taq PCR Master Mix, 0.25 μ l of sense primer, 0.25 μ l of antisense primer, and 23.5 μ l distilled water. The mixture was amplified in a DNA thermal cycler, Touchgen (Tech Limited, England), with the following profile: 94°C for 3 min followed by 33 cycles of 94°C for 1min, 53°C for 1min, and 72°C for 1 min with a final extension step at 72°C for 10min. The PCR products were analyzed using EC250-90 electrophoresis apparatus at 93 V, 500 mA for 1 h and 15 min on a 1.7% agarose gel (IBI agarose, Eastman Kodak Company), 100 ml x 0.5 TBE (Tris-borate-EDTA). A 100-1500 bp DNA ladder (Invitrogen) was co-electrophoresed to estimate the size of the amplified product. The gels were stained with 20 μ l ethidium bromide (10 mg/ml), which attaches to DNA and fluoresces under UV light.

Immunostaining for CAR

RINm5F cells were cultured as monolayer on culture slides (Becton Dickinson Labware, France), RCC and human islets were added to slides pre-treated with poly-L-lysine, before they were fixed in acetone at 4°C. Monoclonal antibodies (Mabs), rose in mice, directed against the CAR (kindly provided by Dr Michael Lindberg, Kalmar College, Sweden) were added to the slides. After washing of the slides, binding of primary antibodies was visualized by the addition of PicTure-Plus Kit (Zymed Laboratories Inc. San Francisco, CA, US) containing a polymer conjugate of horseradish peroxidase HRP and Fab fragments (Reagent A), which reacts with substrate AEC single solution chromogen (Reagent B). The presence of peroxidase is revealed by addition of substrate-chromogen solution. Peroxidase will catalyze the substrate (hydrogen peroxide) and convert the AEC single solution chromogen to a red deposit; which demonstrates the location of the antigen.

RESULTS**Expression of rCAR mRNA**

To visualize the rCAR mRNA in pancreatic β -cells of murine (Rat) origin, the RINm5F (Rat insulinoma cells) cultured in two different ways, the RINm5F cells as monolayers and RCC (RINm5F cell clusters) cultured as free floating cell clusters were used. The expression of rCAR mRNA was shown by RT-PCR (Reverse Transcription – Polymerase Chain Reaction) in both RCC and RINm5F cells, using rCARs and rCARa primers. The PCR products were visualized on an agarose gel with bands of the correct size. In Figure 2a and b exemplifies the results of the amplification by gel electrophoresis. The amplification products of RCC and RINm5F cells appear with a size of 200bp long.

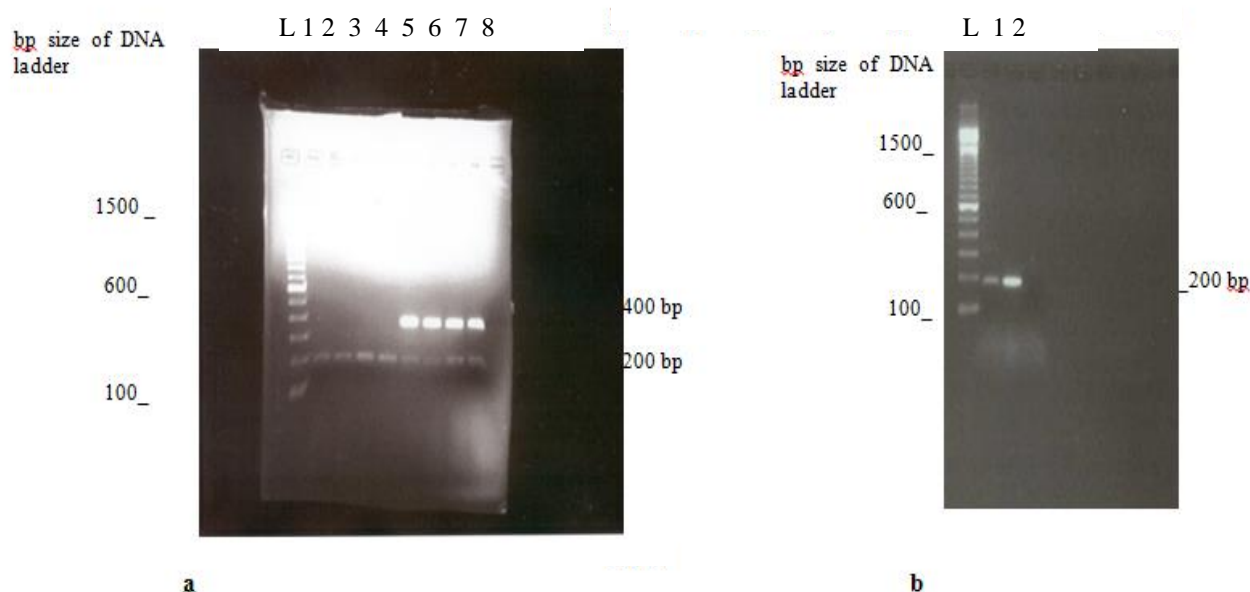


Fig-2 A: Electrophoretic analysis of the PCR products of RCC. Row 1-4 from the left shows the RT-PCR product from RCC samples, and the next four rows 5-8 are the same samples amplified together with rat β -Actin primers (RT-PCR product). The first row from the left is the DNA ladder showing bands between 100 to 1500 bp from the bottom. **b)** Electrophoretic analysis of the PCR products of RINm5F cells. Row 3 from the left show the RT-PCR product from RINm5F cells cultured as monolayer. The first row from the left is the DNA ladder showing bands between 100 to 1500 bp from the bottom

Expression of hCAR mRNA

The hCAR mRNA in human pancreatic β -cells was expressed by RT-PCR (Reverse Transcription–Polymerase Chain Reaction) of mRNA extracted from human islet cells, using hCARs and hCARa primers. The PCR products were visualized on an agarose gel with bands of the correct size. In Figure 3 exemplifies the results of the amplification by gel electrophoresis. The amplification products of human islet cells appear with a size of 200bp long.

Internal control

The internal control Rat β - Actin was amplified and shown by the gel Electrophoreses. According to the primers described in table 1, the primer pair rat β - actin(a) and rat β - actin(s) would give rise to 581 bp long amplification product. While the amplification product gave a 400 bp product when cDNA from both RCC and RINm5F cells were amplified (Figure 2a and b). The amplification of human β - Actin resulted in a 350 bp product (Figure 3).

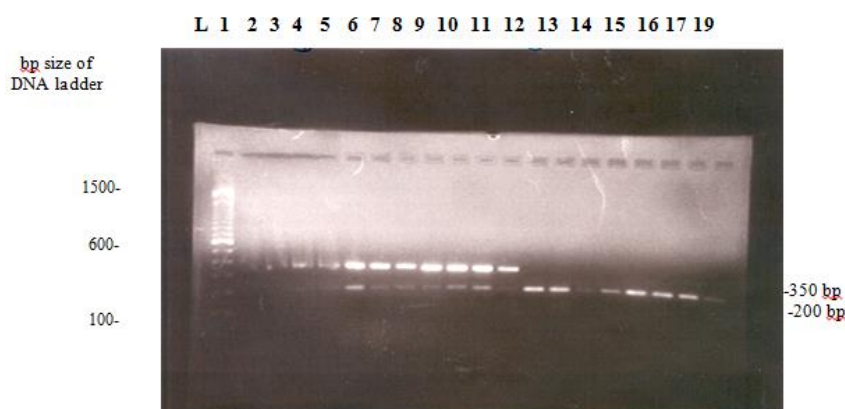


Fig-3: Electrophoretic analysis of the PCR products of human islet cells. Rows 12-19 from the left shows RT-PCR product from human islets samples, and the rows 5-11 are the same samples amplified together with human β -Actin primers. The first row from the left is the DNA ladder showing the bands between 100 to 1500 bp from the bottom

Immunostaining

RINm5F cells cultured as monolayer on culture slides, RCC and human islets added to slides pre-treated with poly L- lysine were fixed in acetone before a monoclonal antibody against CAR was added to the slides. After washing of the slides, binding of primary antibody was visualized by the addition of polymer conjugate of horseradish peroxidase HRPs and Fab fragments, which reacts with substrate AEC single

solution chromogen. The immunostaining for CAR, performed on RCC, revealed cells positively stained to varying extent (Figure 4a). The RINm5F cells cultured as monolayer were not stained at all (Figure 4c). RCC are also shown without addition of the primary antibody as a negative control Figure 4b. Despite mRNA CAR expression in RCC and RINm5F cells cultured as monolayer the protein was only expressed on the RCC.

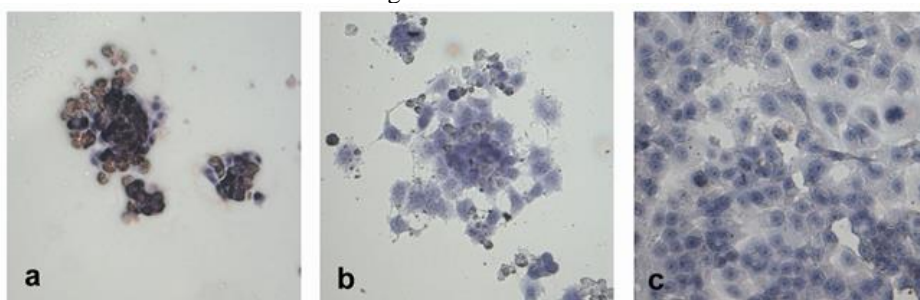


Fig- 4: Immunostaining for CAR (x200). a: RCC stained red positive for CAR (red positive); b: RCC without addition of the primary antibody (negative control); c: RINm5F cells stained for CAR (blue negative)

DISCUSSION

In the present study I analysed the expression of the Coxsackie – Adeno virus receptor (CAR) on insulin producing cells of two different origins. By the use of RT-PCR it was shown that CAR mRNA was expressed in RCC, RINm5F cells, and human pancreatic islet cells. However, when the RT-PCR results were confirmed by immunostaining, only the RCC showed expression of CAR. The RINm5F cells were not positively stained for CAR, suggesting that the protein is not expressed in the cells although the mRNA was expressed. From previous studies CAR may be a component of the tight junction between polarized epithelial cells [9]. Therefore, it is possible that CAR is in the cell-cell contact when the cells are in the monolayer form. Another explanation is that the CAR mRNA is translated in the RCC but not in the RINm5F monolayer cells. Alternative RNA splicing shown to produce three CAR isoform soluble proteins, which lack the transmembrane region [4]. Therefore, the CAR mRNA in the RINm5F monolayer cells might be expressed as a soluble form which can be detected only by expression of the protein not translated as a receptor and identified by immunostaining.

In previous studies CVB has been shown to replicate in RCC while no replication was detected in RINm5F cells [5]. This would be expected if the RINm5F cells did not express CAR as shown by the immunostaining. Since CAR mRNA expression is detected in RINm5F cells by RT- PCR, and immunostaining failed to detect its expression in the cells it might be that CAR in the latter is localized to the tight junction [9]. Another

possible reason could be the different growth pattern of the cells either cultured flat on the slide or forming RCC. The use of β -actin as a internal control when measuring r CAR mRNA resulted in a band of 400 bp, instead of the expected 581 bp. The human internal control β -actin band was of the expected size and I have no explanation for this discrepancy of the rat β -actin mRNA. I am planning to sequence the amplicon to confirm its identity. If CAR is expressed on human pancreatic islet cells it is possible that infection with CVB could induce type 1 diabetes (T1D). There are several studies that show that CVB infection might be associated with T1D [29-35, 20, 36, 37]. RINm5F cells have been used as a model of T1D, since the study of those cells in vitro might indicate what happens to normal β -cells in vivo [38]. Therefore, it is of a real importance that they express the appropriate receptor. The RIN- cell line cultured as monolayer might not be the ideal model for human T1D.

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