The CD24 Protein Inducible Expression System Is an Ideal Tool to Explore the Potential of CD24 as an Oncogene and a Target for Immunotherapy in Vitro and in Vivo*

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Background: It is critical to tightly regulate gene expression to study its biological role.

Results: A tetracycline-dependent CD24 expression system was successfully implemented. An efficient study of its potential as an oncogene and a target for immunotherapy was performed.

Conclusion: This is a valuable tool to study CD24 pathogenesis and novel treatment options for CD24-expressing malignancies.

Significance: The CD24 inducible expression system allows accurate analysis of its role and function.

CD24 is a cell surface, heavily glycosylated glycosylphosphatidylinositol-anchored mucin-like protein that is overexpressed in various human malignancies. To accurately analyze CD24 function and dissect its biological role in a defined genetic background, it is critical to tightly regulate its expression and be able to turn it on/off in a restricted environment and at a specific time. The tetracycline-induced expression system is most promising as it exhibits such regulation, lack of pleiotropic effects, and high and rapid induction levels. To evaluate the oncogenic and immunotherapeutic potential of CD24 by applying the Tet-On system, the human CD24 gene was cloned downstream to two tetracycline operator sequences, resulting in pCDNA4/TO-CD24, which was then transfected into tetracycline (Tet) repressor-expressing cells (293T-REX), allowing tight on/off regulation, thereby resulting in a very low background or leaky CD24 expression. Selected clones were chosen for further studies and characterized in vitro and in vivo, and several treatment modalities were examined. In addition, the role of CD24 in promoting cell proliferation and tumor growth was studied. The tetracycline-dependent system was successfully implemented. Tetracycline treatment induced CD24 expression in a dose- and time-dependent fashion, which was abrogated following treatment with anti-CD24 monoclonal antibodies (mAbs). CD24-induced expression led to an increased proliferation rate that was inhibited by mAb treatment. In vivo, significantly larger tumors were developed in tetracycline-fed mice. The CD24 Tet-On system is a good model to unravel the role and underlying CD24 pathogenesis in vivo. This valuable tool allows the successful study of novel treatment options, whose effectiveness depends on the CD24 expression level. This set of experiments supports CD24 oncogenic properties.

CD24 is a small, heavily glycosylated mucin-like cell surface protein anchored to the membrane via glycosyl phosphatidylinositol (1). CD24, first attributed as a differentiation marker for B cells, is involved in lymphocyte maturation (2, 3), regulates the proliferation of neuronal precursors (4), and was shown to be involved in the regulation of homeostatic cell renewal (5). CD24 is known to be overexpressed in various human malignancies, both solid and hematological (6, 7), and is usually tied with a more aggressive course of the disease (1, 8, 9).

In our previous studies, we had shown the importance of CD24 in the multistep process of colorectal cancer carcinogenesis, already at the early stage of adenoma (10). Stringently controlled conditional expression systems are crucial for the functional characterization of genes. The development of an expression system with the ability to control and regulate the level and duration of expression is useful for elucidating the functions of CD24 and its impact in the pathogenesis process. The tight regulation of CD24 expression was achieved by the widely accepted inducible Tet-On system. The tetracycline-controlled transcription activation system has been already successfully employed in a variety of eukaryotic cells, including mammals (11, 12), plants (13), and yeast (14) cells, and at the organism level, in mice (15, 16), plants (17), and Drosophila (18).

The CD24 Tet-On system allows a precise study to evaluate the role of CD24 and its function, partly because it is a defined system with minimal noise and background. Each experiment was performed on the same cell population; therefore, there are no unknown differences between the control and the experimental groups that can enhance the heterogeneity of the results. Therefore, this model system may also serve to effectively evaluate the effectiveness of new immunotherapy options against CD24-expressing cells.
EXPERIMENTAL PROCEDURES

Materials

All reagents were purchased from Sigma (Rehovot, Israel), unless otherwise stated. Secondary horseradish peroxidase-conjugated antibodies were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). EZ-ECL detection kit and cell culture supplements were from Beit-Haemek, Israel.

Methods

Establishment of CD24-expressing Cells

Plasmid Construction—Initially, a DNA fragment coding for a full-length human CD24 was amplified by PCR using the plasmid pCMV-SPORT6-CD24 as a template using primers Kozak-HindIII-CD24-F (5'-CTGGAAGCTTGCC-ACCATGGATGGGCAGAGCAATGGTGGC-3') and XbaI-CD24-R (5'-TCATCTAGGTATTAAGAGTAGAGATGC-AGAAG-3'). The PCR product was digested by HindIII and XbaI and inserted into the pcDNA4/TO (pcDNA4 tetracycline operator) plasmid, downstream to two tetracycline operator sequences, TetO2, which was cleaved with the same enzymes. pcDNA4/TO-CD24 was transfected into 293T-REx stable cline-regulated mammalian expression system (19, 20).

Two different 293T-REx-CD24 clones were examined. Establishment of CD24-expressing Cells

The T-REx System—The T-REx system is a tetracycline-regulated mammalian expression system (19, 20). pcDNA4/TO-CD24 was transfected into 293T-REx stable cells expressing the tetracycline repressor from the pcDNA6/TR vector (Invitrogen), using the calcium phosphate transfection method. 48 h after transfection, the cells were seeded into DMEM medium supplemented with 10% fetal bovine serum (FBS), containing the selectable marker Zeocin (InvivoGen, 100 μg/ml). Several clones were isolated and characterized.

CD24 Binding Assay

Evaluation of CD24 induction was done by specific binding of anti-CD24 mAb using flow cytometry. Approximately 1 × 10^6 293T-REx stable transfected cells were used in each experiment. After trypsinization, the cells were washed in FACS buffer (10% FBS, 0.01% sodium azide in ice-cold PBS) and fixed with 2% formaldehyde (in PBS) for 15 min. Then, 100 μl of 10 μg/ml anti-CD24 mAb were added for 30 min at room temperature. Following washes, FITC-labeled goat anti-mouse antibodies diluted 1:100 in FACS buffer were added for 30 min at room temperature and protected from light. Detection of bound antibodies was performed on a FACSCalibur (BD Biosciences), and results were analyzed with the CellQuest program (BD Biosciences).

Viability Assay

293T-REx stable transfected cells were seeded in 96-well plates (2 × 10^4 cells/well) in complete medium. On the next day, ZZ-PE38, WT PE, SWA11, ZZ-PE38 and IgG-PE38 were added to the cells in quadruplicates at various concentrations. 48 h later, the medium was replaced by immunocomplex-free medium (100 μl/well) containing 1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent and incubated for 2–4 h. MTT-formazan crystals were dissolved by the addition of acid isopropanol alcohol (0.04 N HCl in isopropanol alcohol). Absorbance at 570 nm and a reference wavelength of 690 nm were recorded on an automated microplate reader. IC_{50} was determined as the immunotoxin concentration that caused 50% death when compared with untreated cells.

Xenograft Model for Measuring in Vivo Tumor Development

Athymic nude mice (Harlan Laboratories, Rehovot, Israel) were housed in sterile cages and handled with aseptic precautions. The mice were fed ad libitum. To test the tumor-promoting potential of CD24, exponentially growing 293T-REx-CD24 cells were harvested and resuspended at a final concentration of 5 × 10^6 or 10 × 10^6 cells per 0.2 ml of PBS per injection. The cells were injected subcutaneously at two sites on the flanks of the mice. The mice were weighed, and tumor growth was measured twice weekly with a caliper; volume was calculated as containing 5% FBS. On the next day, the serum was reduced to 2.5% with or without 1 μg/ml tetracycline. Every 3 days, cells were collected and counted from three wells to assess the growth rate.

Preparation of ZZ-PE38 Fusion Proteins

The armed anti-CD24 mAb is a novel antibody-toxin immunon conjugate where the targeting moiety is an anti-CD24 SWA11 mAb, whereas the toxic moiety is a truncated form of the Pseudomonas exotoxin (PE)³ (Shapira et al. (21)). The expression and purification of the wild-type (WT) PE, ZZ-PE38, and the fusion proteins, SWA11/IgG-ZZ-PE38, were performed as described by Shapira et al. (21) Briefly, the pET22b-ZZ-PE38 plasmid (22), which carries an in-frame fusion of ZZ to PE38, was designed for the expression of soluble ZZ-PE38 fusion protein in the Escherichia coli periplasm. The Fc-binding protein ZZ is a duplication of mutated B domain of Staphylococcus aureus protein A, which is quite effective at binding the Fc domain of mouse IgG2a immunoglobulins (22, 23). The conjugation of SWA11 and normal IgG (control) antibodies to ZZ-PE38 fusion protein was performed as follows. Antibodies, diluted in PBS, were mixed with ZZ-PE38 in PBS (3-fold molar excess of ZZ-PE38 over IgG) for 16 h at 4 °C. Separation of excess ZZ-PE38 from the IgG-ZZ-PE38 complex was performed by applying the sample onto a 25-ml Superdex 200 size exclusion column (GE Healthcare) as recommended by the manufacturer. The resulting immunocomplexes were SWA11-ZZ-PE38 and IgG-ZZ-PE38.

³ The abbreviations used are: PE, Pseudomonas exotoxin; Tet, tetracycline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
At the end of the experiment, the mice were sacrificed after anesthesia by cervical dislocation, and the tumors were excised. The study was approved by the Institutional Committee for Animal Welfare at the Tel Aviv Sourasky Medical Center.

RESULTS

Establishment of an Inducible CD24 Tet-On Expression System—Stable clones were established, and their CD24 gene tetracycline inducibility was evaluated. Two representative clones (clones 13 and 15) with high inducible expression were chosen for further studies. Tetracycline increased significantly the expression of CD24, as can be seen in Western blot (Fig. 1). These results were confirmed using flow cytometry for membrane CD24 levels (data not shown). In the absence of tetracycline, the clones expressed only low basal level of CD24, indicating that CD24 expression is tightly regulated without leakiness.

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CD24 Expression Level Is Regulated by Tetracycline in a Dose- and Time-dependent Manner—293T-REx-CD24 cells were treated for 24 h with different concentrations of tetracycline (0, 0.01, 0.05, 0.1, and 1 \( \mu \)g/ml). CD24 expression level was proportional to the tetracycline concentration as can be seen by Western blot (Fig. 2A) and FACS analysis (Fig. 2B).

The CD24 expression level was increased in a time-dependent manner following exposure to 1 \( \mu \)g/ml tetracycline for 0, 4, 6, 16, 24, 30, or 48 h. Its expression level increased from a baseline to a maximal level as shown by time lapse, as analyzed using TINA20 software (Fig. 2C).

Increased Expression of CD24 Increases Colony Formation and Cell Proliferation—Fig. 3A shows representative experiments that demonstrate the increase in growth rate following induction of CD24 expression (squares). Fig. 3B demonstrates qualitatively, by crystal violet staining, the different growth rates between induced and uninduced cells. Increased proliferation rate is correlated to the tetracycline concentration (Fig.
In addition, the plating efficiency was 3-fold higher when CD24 expression was triggered (Fig. 3D).

**Susceptibility of CD24-expressing Cells to Anti-CD24-targeted Therapy, Using Naked and Armed Antibodies**—CD24-induced expression levels were examined after treatment with anti-CD24 mAb. The expression levels decreased following antibody treatment, as can be demonstrated by the Western blot (Fig. 4A) and FACS analysis (Fig. 4B).

In addition, the cells were grown in the absence or presence of various concentrations of anti-CD24 mAb for 48 and 96 h. The growth of the induced cells was inhibited in a dose-dependent manner (Fig. 4C), whereas no significant effect was demonstrated in the uninduced cells (data not shown).

We evaluated the cytotoxicity of the armed anti-CD24, SWA11-ZZ-PE38 immunotoxin, when compared with the WT PE toxin and with a nonspecific immunotoxin, IgG-ZZ-PE38. According to the cell killing experiments, CD24-induced expression induced sensitivity to the SWA11-ZZ-PE38 immunotoxin treatment (Fig. 5A) with an IC\textsubscript{50} value of 20 ng/ml. Conversely, no sensitivity was observed toward the control nonspecific immunotoxin (Fig. 5B), IgG-ZZ-PE38, nor to the individual components of the immunotoxin conjugate (Fig. 5D). As can be seen in Fig. 6, the therapeutic window between the induced and uninduced cells was opened only in the cells that were treated with the SWA11-ZZ-PE38, emphasizing the specificity of this targeted tool and its dependence on CD24 expression.

As expected, the WT PE, which has its own cell binding domain, is able to kill the cells regardless of CD24 expression (Fig. 5C). This is demonstrated by the similar IC\textsubscript{50} values of the induced and uninduced cells (10 ng/ml for both) and resulted in no therapeutic window. However, the IC\textsubscript{50} values of SWA11-ZZ-PE38 in inducible CD24-expressing tumor cells were similar to those of the WT PE toxin (10 ng/ml), emphasizing the potency of the immunocomplex against CD24-expressing tumor cells.

**CD24 Promotes Tumor Growth in Vivo**—Two experiments were performed. In the first one, the cells were grown with or without 1 µg/ml tetracycline 1 week prior to injection into nude mice. Each mouse was injected subcutaneously with two clones;
clone 13 was injected in the right flank and clone 15 was injected in the left flank, to rule out a clone-specific effect. In total, there were four groups as described in Table 1. Mice from group 3 developed tumors in both flanks (Fig. 6A), whereas no tumors were developed in group 4 (Fig. 6B). Group 1, which was injected with a smaller amount of cells, also developed tumors but after a longer period of time (~1 month). Still, the uninduced mice did not develop any tumors.

Tumors from both groups were removed, and CD24 expression was analyzed by Western blotting. All the tumors expressed high levels of CD24, similar to those obtained with induced cells in vitro (data not shown).

The second experiment included two groups with multiple subjects (six mice in each group). All the mice were injected subcutaneously with $10^5$ cells. In this experiment, the cells were grown with or without $1 \mu g/ml$ tetracycline 48 h prior to the injection. Fig. 6C shows that CD24 expression indeed promotes tumor formation and development, as demonstrated in the previous experiment. As expected, 293T-REx™ cells also developed tumors, but at a slower growth rate.

Before cell implantation, samples from the growing cultures were analyzed by Western blot to confirm the induction of CD24 expression (Fig. 6D). In addition, 1 month after cell injection (at the end of the experiment), tumors were removed, and CD24 expression levels were examined (Fig. 6E). Expression was induced by tetracycline in the tumor and was increased when compared with the control mice that received only sucrose. There was a close association between CD24 expression level and tumor volume.

**DISCUSSION**

A proof of concept of the Tet-On inducible expression system is reported herein. This system can serve as an important research tool to unravel and clarify the functional roles of the CD24 gene and its importance in the multistep carcinogenesis process. Indeed, it has been confirmed that CD24 is a potential oncogene.

Usually, to study these goals, human cancer cell lines expressing a specific gene are compared with a different cell line that does not express the gene. Obviously, there are many more known and unknown differences between these cell lines that can enhance the diversity of the outcome analysis due to unknown factors embedded in the cells, and their effect and/or impact cannot be discarded. Therefore, it is difficult to isolate the effect of a specific gene.
It is apparent that a stable cell line that overexpresses a specific gene, such as the CD24 gene demonstrated in this study, can be compared with the one that contains only the empty vector. Representative clones can be chosen for further studies, but one should keep in mind that different clones are, in fact, different cell populations with variations between the stable clone and the empty vector control. In addition, constitutive and forcefully high expression of the protein may become a burden for the cells, leading to a change in their behavior. The inability to control the level of expression is another significant disadvantage of these constitutive expression systems. The inducible expression system may overcome these obstacles and would serve as an ideal tool for unraveling the downstream effectors of the gene and shedding light on the role of the gene, its function, and its potential as a therapeutic target.

Regulation of gene expression in a temporal and spatial manner provides a useful tool for the study of mammalian gene function particularly during oncogenesis. The Tet regulatory systems are currently the most widely used models for conditional gene expression. Maximal expression levels in these systems are very high and comparable with the maximal levels reachable from a constitutive and strong mammalian promoter such as CMV (24). Indeed, the level of CD24 protein following exposure to tetracycline was comparable with the level of the protein after transient expression (data not shown).

The current study demonstrates the effect of increased expression of CD24 in 293T-REx™ cells, where its expression is regulated by tetracycline. It was confirmed that inducible expression of CD24 resulted in a more malignant phenotype. The inducible cells proliferated faster and demonstrated an increased saturation density and plating efficiency. Most importantly, they were more tumorigenic in vivo when injected subcutaneously to nude mice. These results are compatible with numerous publications linking CD24 with rapid cancer cell growth (25–27).

This system is very sensitive, and the control of CD24 can be achieved in a time- and dose-dependent manner. These characteristics of the system are very important as they can carefully dissect the oncogenic potential of CD24. The very low background of CD24 when the cells were not exposed to tetracycline confirms the non-leakiness of this inducible system.

The potential for targeting CD24 in cancer therapy seems promising as CD24 is overexpressed in many human cancers (9, 28). Using this system, we were able to study new treatment modalities for CD24-expressing cancer cells where the only difference between the treated and untreated cultures is the level of CD24 expression. We were able to compare the effects of several existing mAbs targeting CD24, e.g. SWA11 (29), as well as novel immunoconjugates that were produced in our laboratory (Shapira et al. (21)). Down-regulation of CD24 expression using mAbs resulted in decreased expression level and growth inhibition of the induced cells in vitro. In addition, specific delivery of more potent treatment modalities, such as the SWA11-ZZ-PE38 immunotoxin, effectively killed the cells depending on CD24 expression levels. In contrast, the WT Pseudomonas exotoxin, which possesses its own cell binding domain, enters into mammalian cells via a receptor-mediated endocytic pathway through binding to the multiligand receptor found in most cultured cells (30). Therefore, it kills the cells regardless of CD24 expression.

Above all, the system proved itself in vivo. HEK293 cells are tumorigenic, but it was clearly shown that up-regulation of
CD24 expression increases cell tumorigenicity in vivo. Nude mice that were implanted with the cells and received the inducer in their drinking water developed impressive tumors in contrast to the uninduced mice. Not only was tumor incidence significantly lower in the control group, but the rate of tumor growth was significantly slower as well, resulting in smaller tumor volume. These results provide strong evidence for the potential oncogenicity of CD24.

In summary, the Tet-controlled CD24 expression system has a tight and dose-dependent inducibility for tetracycline. This further suggests that CD24 is a tumor-promoting agent that markedly enhances tumor development. This system is a potential tool to study the functional role of CD24 and evaluate novel therapeutic modalities against it.

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