Relationship of IL-19 to the Metastatic Potential of a Lung Tumor

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Abstract
Interleukin 19 (IL-19), an anti-inflammatory and immunosuppressant cytokine, has been shown to affect the function and viability of lung fibroblasts, and has been implicated in lung cancer metastasis. The purpose of this research was to compare gene expression profiles of IL-19 genes (through the collection of RNA isolates) between two lines of mice, an original tumor line (DA-3/TM), and a surgically resected metastatic tumor line (DA-3/TM-Mets). A battery of laboratory procedures were used to study metastasis of cancer from a primary tumor cell line, DA-3/TM, and from a metastatic site, DA-3/TM-Mets. Metastatic lesions in animal models were isolated, cultured, and analyzed in order to observe the changes between the primary and metastatic line. Both PCR and Western Blots were performed to verify the initial findings from the metastatic lesions of mice. It was found that DA-3/TM-Mets, had more nodule metastasis throughout the lungs of BALB/c mice compared to that of DA-3/TM. A gene array performed showed that the key difference was IL-19, which was 30X higher in DA-3/TM-Mets than in DA-3/TM. This suggests a significant difference at the gene level. However, it is important to develop a new model to look at these tumor cell lines at the protein level in order to observe if this difference holds true. The data acquired shows promising results. Higher concentrations of IL-19 in the DA-3/TM-Mets line may lead to the conclusion that IL-19 is exhibiting immunosuppressive activity, which does not allow the BALB/c mice’s immune system to fight off a tumor, resulting in their death. Therefore, there appears to be a direct correlation between the metastasis of cancer and the protein IL-19’s immunosuppressive effects.

INTRODUCTION
IL-19, an anti-inflammatory and immunosuppressant cytokine, has been shown to affect the function and viability of lung fibroblasts, and has been implicated in lung cancer metastasis. To date, various lines of research have employed gene array in order to understand the genetic basis of oncogenic mediators. The purpose of this research was to compare gene expression profiles of IL-19 genes (through the collection of RNA isolates) between two lines of mice, an original tumor line (DA-3/TM), and a surgically resected metastatic tumor line (DA-3/TM-Mets).

The focus of the project was to explore whether or not IL-19 was present in higher concentrations in DA-3/TM-Mets than DA-3/TM using Polymerase Chain Reaction tests along with Western Blots. If there was a higher concentration of IL-19 in DA-3/TM-Mets than in DA-3/TM, then it would explain the fact that IL-19 may exert immuno-suppressive effects. These immunosuppressive effects would explain why the Balb/c mice with the metastatic tumor cell line, DA-3/TM-Mets, died sooner than those mice with the primary tumor cell line, DA-3/TM.

METHODS

MONITORING MEAN TUMOR VOLUME/SURVIVAL ANALYSIS-
The DA-3/TM and DA-3/TM-Mets cells were cultured and then subsequently added subcutaneously into 5 mice each. Approximately 1 week after these mice were subcutaneously injected, the mice were palpated to see whether tumor growth had occurred. When tumor growth had occurred, the tumor was measured based on its length and width dimensions. Once the data had been retrieved, a specific calculation was done to monitor the tumor volume. A second set of mice were also injected subcutaneously with DA-3/TM and DA-3/TM-Mets and were followed until death. The lungs were extracted from these mice and analyzed as can be seen in Figure 3.

RNA ISOLATION
The DA-3/TM and DA-3/TM-Mets cells were cultured and the tumor cell lysates were retrieved. RNA was then isolated.
from DA-3/TM and DA-3/TM-Mets cells using the TRI-Reagent protocol. It was very important to use gloves when handling RNA. After the cell lysates were retrieved, they were homogenized, which could not be more than 10% of the TRI Reagent volume. Following homogenization, the sample was incubated for 5 minutes at room temperature. Bromochloropropane was added and the sample was vortexed for 15 minutes. The sample was then incubated for 15 minutes at room temperature. Following incubation, the sample was centrifuged at 12,000 g for 15 minutes at 4°C. Two distinct layers formed, one containing the aqueous RNA layer. This aqueous RNA layer was transferred to a new tube. 0.5 mL of isopropanol was added to this aqueous layer per mL of TRI-reagent. The sample was incubated for 10 minutes at room temperature. Then the sample was centrifuged at 12,000 g for 8 minutes at 4°C.

An RNA pellet was formed on the bottom of the tube. The RNA pellet was then washed using 1 mL 75% ethanol. Once again, the pellet was centrifuged at 7,500 g for 5 minutes at 4°C. The pellet was allowed to air dry for 5 minutes before being dissolved. Once dissolved, the RNA concentrations were checked before proceeding to make cDNA. The TRI-Reagent protocol for RNA isolation was used in both the gene array and PCR.

**GENE ARRAY**

The gene array was conducted on RNA, using the SuperArray Oligo GEArray system. The TRI – Reagent protocol was used for isolating the RNA from DA-3/TM and DA-3/TM-Mets tissue samples. The ArrayGrade Total RNA Isolation kit was used for RNA isolation of cultured cells. Both the TRI-Reagent Protocol and ArrayGrade Total RNA Isolation kit are a part of the TrueLabeling-AMP 2.0 protocol. Each kit contains: aTrueLabeling Primer, RNase Inhibitor, cDNA synthesis enzyme mix, 5X cDNA synthesis buffer, RNA polymerase enzyme, 2.5X RNA polymerase buffer, and RNase-free water.

The first step was to make the annealing mixture, which contained isolated RNA from the tumor cell lines, 1 microliter of TrueLabeling Primer, and RNase-free water, in which the total sample is adjusted to a total of 10 microliters. This sample is then incubated at 70°C for 10 minutes. The second step was to synthesize cDNA. So 4 microliters of 5X cDNA synthesis buffer, 4 microliters of RNase-free water, 1 microliter of RNase inhibitor, and 1 microliter of cDNA synthesis enzyme mix were added. The sample was then incubated at 42°C for an hour and 75°C for 5 minutes. The sample was cooled to 37°C. Following this step, it becomes important to amplify the sample. For each array, 16 microliters of 2.5X RNA Polymerase buffer, 2 microliters of Biotin-UTP, and 2 microliters of RNA Polymerase enzyme were added to the sterile PCR tube. The sample was incubated at 37°C overnight. The water was discarded from the PCR tube and 0.75 mL of hybridization solution containing the probe was added. The solution was incubated at 60°C overnight with continuous agitation. The membrane was washed once with 2X SSC and 1% SDS for 15 minutes at 60°C. The membrane was washed again with 0.1X SSC and 0.5% SDS for 15 minutes at 60°C. The tube was then vortexed after adding each wash solution. A chemiluminescent detection kit was used in order to acquire the image of the gene array.

**POLYMERASE CHAIN REACTION**

RNA concentration was determined, and the first strand cDNA (complimentary DNA) was reverse transcribed using the Fermentas first strand cDNA kit. This kit contained oligo(dT)_18 primer,. Ribolock RNase Inhibitor, M-MuLV Reverse Transcriptase, and your RNA sample. Once cDNA is synthesized, the Fermentas PCR kit was used in preparation of PCR. The PCR kit contained nuclease-free water and 2X PCR master mix. Then the primers are added along with the cDNA to get a total of 25 microliters in the test tube. Then for each cDNA of DA-3/TM and DA-3/TM-Mets, IL-19 was added and thus amplified to show where the concentration was greater. This was done using a PCR machine, heating and cooling the sample for 40 cycles. The samples were then dyed and gel electrophoresis was conducted in an agarose gel. The results were analyzed (Figure 9).

**WESTERN BLOTS**

The western blot was performed according to BIO-RAD #161-0772 protocol. The DA-3/TM and DA-3/TM-Mets cells were cultured, and the tumor cell lysates were retrieved. The flasks containing the lysates were shaken at 4°C for fifteen minutes; the tumor cell lysates were then transferred to tubes, vortexed, and placed on ice for twenty minutes. The lysates were then centrifuged and the supernatants, which contained the protein extracts, were transferred to new tubes and stored at -80°C. Protein concentration was retrieved. Fifty micrograms of protein were bound to the 10% SDS polyacrylamide gels and then transferred to nitrocellulose paper. The nitrocellulose was blocked at room temperature in nonfat dry milk in TBS-T. The nitrocellulose was then rinsed in TBS-T and incubated
overnight in a primary antibody at 4°C. The blots were then washed for two 30 minute periods in TBS-T. The blot was incubated at room temperature with horseradish peroxidase conjugated monoclonal Anti-Mouse IL-19 antibody (R&D systems) for 1.5 hours. The blots were then washed four times for 10 minutes each in TBS-T and incubated in a chemiluminescent substrate for 5 minutes (8). The results could be seen by exposing the blots to CL-Xposure film (see Figures 10 and 11). The film was scanned and the results analyzed.

RESULTS

MEAN TUMOR VOLUME / SURVIVAL ANALYSIS/ METASTASIS

When referring to Figures 5 and 6, there appears to be no major difference in the size of the tumors in those mice injected with DA-3/TM compared with those mice injected with DA-3/TM-Mets. It is important to remember that the size of the tumor does not correlate to how well it could metastasize. Therefore, the fact that there appears to be little difference in the size of the tumors between DA-3/TM and DA-3/TM-Mets may strengthen the argument that the key difference between the tumors is the metastasis capability of DA-3/TM-Mets. Another difference between the tumor cells was survival time. Those mice that were subcutaneously injected with DA-3/TM-Mets died by 8 weeks post injection; whereas, after 8 weeks those mice injected with DA-3/TM were all alive. When the lungs were removed from the DA-3/TM and DA-3/TM-Mets mice around the 7th week post injection, the results can be seen in Figure 7. The DA-3/TM-Mets mice had much more nodule metastasis in the lung than the DA-3/TM mice. The white spots on the lungs in Figure 5 shows the number of visible nodules, which were recorded as 53, 114, and 53 respectively of the three DA-3/TM-Mets mice that were compared. Since there seems to be a wide gap between the 1st/3rd mice (53 nodules each) vs. the 2nd mouse (114 nodules), more testing should be conducted. However, there definitely appears to be a correlation between DA-3/TM-Mets mice and lung metastasis.

GENE ARRAY

Why this metastasis is occurring in mice subcutaneously injected with DA-3/TM-Mets compared with that of DA-3/TM had to be investigated on the gene level. The chemokine and chemokine receptor assay (Figure 8) shows some genetic variations at the RNA level. It can be seen that the chemokine, IL-19, is 30 fold higher in the DA-3/TM-Mets cell’s RNA than the DA-3/TM cell’s RNA. To the naked eye, it can be seen that IL-19 shows up on the DA-3/TM-Mets assay, shown in red, but not on the DA-3/TM assay, shown in blue. This gave the laboratory a new model to look at that had never been done before. The goal now was to verify this gene array and continue to look at the protein IL-19 and its possible effects on metastasis in Balb/c mice. A PCR test was used to verify the gene array. If this test showed similar results, then it was important to investigate this protein, IL-19, on the protein level.

PCR

The initial data in PCR lacked supportive results when comparing to the gene array data shown in Figure 6. However, improving on PCR techniques proved that this initial data contained human error. When isolating RNA and reverse transcribing cDNA, pipetting the correct amounts of different solutions into the sample was a challenge without contaminating the pipette tips. However, allotting the appropriate time to do the experiment along with correcting the pipette issue proved to be successful in my final results, shown in Figure 9. One other challenge that was initially present was that the samples were placed in the PCR machine for twenty-five cycles to begin with. After gel electrophoresis was completed, the gels did not show any bands whatsoever. However, when the number of cycles on the PCR machine was increased to forty cycles, the bands appeared on the gels. Therefore, trial and error along with human error were the cause of deficient results initially.

The data attained when the PCR was done correctly proved to be more favorable. Looking at Figure 9, the IL-19 band is clearly shown in the DA-3/TM-Mets lane, whereas, no band exists in the DA-3/TM lane. This supports the initial gene array, which shows a higher concentration of IL-19 in DA-3/TM-Mets than in DA-3/TM (Figure 6). By evaluating the data in Figure 9, done by PCR, along with the data in Figure 8, by gene array, it appears as though IL-19 is the primary cause of metastasis of DA-3/TM-Mets. However, due to time constraints on the project much more testing should be conducted to render this experiment accurate. Now that the gene array was verified, it was very important to look at what was going on with IL-19 at the protein level.

WESTERN BLOTS

Western blotting is a technique used to evaluate proteins (described in the introduction). The data in Figures 8 and 9 showed a difference in IL-19 between DA-3/TM and DA-3/TM-Mets.
TM-Mets. However, just like PCR, the first few western blots lacked these same differences in DA-3/TM and DA-3/TM-Mets. A couple of reasons exist as to why this might have occurred. The first of these reasons was probably due to human error. The human error involved was most likely due to learning how to pipette correctly and spending sufficient time conducting the experiment. The effect of poor pipetting would be skewed results on the developed films between DA-3/TM and DA-3/TM-Mets. For example, in one particular experiment using the same pipette tip caused contamination of the samples, thus, rendering the experiment null and void. Another possible reason less accurate results were initially attained, could have been due to a bad antibody. Therefore, it was important to try antibodies of different strengths to see which was the most compatible with the experiment. Both of these factors were investigated; more caution was headed when setting up the experiment and when using the pipette, as well as the correct antibody for IL-19 was ordered.

The data attained when more precautionary steps were taken proved to be more favorable when looking at the results. Looking at Figures 10, there is a noticeable larger concentration of IL-19 in DA-3/TM-Mets than DA-3/TM. This can be seen more clearly in a magnified version of the film, Figure 11. This finding means that there appears be some sort of correlation between IL-19 and DA-3/TM-Mets than DA-3/TM. Ultimately, this supports the data attained in Figures 7, 8, and 9. Due to time constraints, more testing needs to be conducted; ultimately, this should allow for a more definitive and accurate conclusion. By strictly evaluating the data attained by western blots, it appears that IL-19 is the cause of immunosuppressive effects seen by the tumor cell line, DA-3/TM-Mets, in Balb/c mice. Thus, this could have been a reason that the Balb/c mice injected with DA-3/TM-Mets had more nodule metastasis and died sooner than the Balb/c mice injected DA-3/TM.

DISCUSSION

Much is known about metastasis in scientific literature today. However, how do the results obtained shown in Figures 7-11 compare to any of those already published? It is now known that IL-19 is a cytokine that was just recently found and had the same α helical structure as IL-10; thus, it was included in the IL-10 family. One scientific research experiment studied the correlation between IL-10 receptor mRNA levels and renal cell carcinoma metastasis. It was found that a higher tumor to non-tumor ratio of IL-10 receptor mRNA’s expression correlated with an overall shorter survival of the patient and a higher metastasis. IL-10 is exerting immuno-suppressive effects allowing the metastases to spread to the kidney and, thus, killing the patients in a shorter period of time. The known immuno-suppressive effects of IL-10 include interruption of T cell proliferation, isotype switching in B cells, and blocks the production of proinflammatory cytokines.

In our laboratory, all five mice had died by the 8th week when being subcutaneously injected with DA-3/TM-Mets. However, no mice had died by the 8th week when being subcutaneously injected with DA-3/TM. It was shown in the results that the key difference between DA-3/TM-Mets and DA-3/TM was a cytokine called IL-19, seen in the gene array (Figure 8). Since IL-19 and IL-10 are in the same family and share a similar structure, it should be deduced that they could possibly be exerting similar effects on the metastasis of their tumor cell lines (IL-10 on the kidney and IL-19 on the lungs).

IL-19 proves to be important in the metastasis of DA-3/TM-Mets to the lungs. The gene array (Figure 8), PCR (Figure 9), and western blot (Figures 10 / 11) all show significant results when compared with each other. These results also correlate to the picture of metastasis in the lungs shown in Figure 7. While the exact mechanism of IL-19 immuno-suppression needs to be studied further in DA-3/TM-Mets, there does appear to be a correlation between the two. However, it remains necessary that additional testing be conducted to confirm the results.

**Figure 1**

Figure 5- This set of first measurements conducted on Balb/c mice showed no significant difference between DA-3/TM-Mets and DA-3/TM tumor cell lines in relation to mean tumor volume.
Figure 2
Figure 6- There was no significant difference between DA-3/TM-Mets and DA-3/TM tumor cell lines in relation to the mean tumor volume.

**DA-3/TM and DA-3/TM-Mets Tumor Growth (Data Set 2)**

![Graph showing tumor growth comparison between DA-3/TM and DA-3/TM-Mets](image)

Figure 3
Figure 7- Metastatic tumor nodules were more numerous in the DA-3/TM lungs (avg. # =73), whereas at nine weeks, DA-3/TM mice showed no signs of metastatic disease.

![Images of lung metastasis](image)

Figure 4
Figure 8- Chemokines and chemokine receptors array map. A reference of the specific locations can be found in the Oligo GEArray protocol.

**Chemokines and Chemokine Receptors Array**

![Chemokine and receptor array map](image)
**Figure 5**

Figure 9- This Polymerase Chain Reaction gel shows an IL-19 band in the DA-3/TM-METS lane, whereas, no band exists in the DA-3/TM lane.

**Figure 6**

Figure 10- Western blot that shows a greater concentration of IL-19 in the DA-3/TM-Mets lane marked in red than in the DA-3/TM lane marked in blue.
Fig. 7

Figure 11- A magnified version of Figure 3 that shows a Western blot with a greater concentration of IL-19 in the DA-3/TM-Mets lane marked in red than in the DA-3/TM lane marked in blue. We know that there is a greater concentration due to a more prominent IL-19.

Western Blots of DA-3/TM and DA-3/TM-Mets (#2)

References

11. 'TRI- Reagent RNA, DNA and Protein Isolation Protocol", file:///C:/DOCUME~1/JOSHUA~1/LOCALS~1/Temp/trireagent.html
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