CERVICAL CANCER-ASSOCIATED HUMAN PAPILLOMAVIRUS 16 E7 ONCOPROTEIN INHIBITS INDUCTION OF ANTI-CANCER IMMUNITY BY A CD4$^+$ T CELL DEPENDENT MECHANISM.

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Key Words: HPV, Cervical Cancer, Oncogene, Immunotherapy, Immune Evasion, Vaccine

Abbreviations: hGH: human growth hormone, HPV: Human Papillomavirus, Ab: Antibody

Abstract

Attempts to develop therapeutic vaccines against cervical cancer have been proven difficult . One of the major causes of the failure is due to the use of the wrong mouse models based on transplantable tumours in testing the efficacy of vaccines. Now that a transgenic epithelial mouse model has been developed to closely mimic cervical cancer, the mechanisms needed to eliminate this type of cancer could be studied. The E7 oncoprotein of Human Papillomavirus (HPV) is the most expressed HPV protein in cervical cancers and its continuous production is essential to maintain the cancerous state and therefore the obvious target in the development of vaccines. Skin grafts expressing the HPV 16 E7 protein (E7 autografts) are not spontaneously rejected from an MHC matched immunocompetent host. Interestingly, simultaneous placement of an MHC mismatched skin (allograft) next to an E7 autograft results in the E7 autograft rejection. However when the allograft also expresses E7, the E7 autograft is rejected more slowly. Autograft rejection requires $CD8^+$ T cells, and is accelerated by removal of $CD4^+$ T cells after placement of the E7 expressing allograft, suggesting induction of an E7 specific $CD4^+$ regulatory T cell population by the E7 expressing allograft. This observation may have implications in designing effective vaccines and immunotherapy against cervical cancers in women.

Introduction

HPV infections, especially types HPV16 and HPV18, are the major cause of cervical cancers with more than 99% of observed tumours containing HPV DNA (1). Prophylactic vaccines against HPV are now available (2). However there is an urgent need to develop a therapeutic vaccine to cure women who already have cervical cancer, and to eradicate HPV-containing cancer/pre-cancer cells from the estimated 10.5% of females worldwide who are already infected with the virus (3). Human cervical cancers produce the HPV-derived E7 oncoprotein whose continuous production is essential to maintain the cancerous state (4). Therefore the E7 oncoprotein is a prime target for anti-cervical cancer therapeutic immunotherapies. In the past, anti-E7 vaccines were formulated based on data from E7-producing transplantable tumours in mouse models (5, 6, 7). However these vaccines failed when tested on cervical cancer patients (8, 9). One of the reasons that the attempts to develop a therapeutic vaccine against cervical cancer have failed in the past is due to the use of the wrong mouse model (E7 producing transplantable tumours) to test vaccine efficacy. The cause of this failure could be that cervical cancers are epithelial in origin and need a different type of an immune response to eliminate the cancer.

To this end, a transgenic mouse model (K14E7) was developed previously in which keratinocytes express HPV-16 oncoprotein E7 under the control of the keratin-14 promoter (10, 11). K14E7 skin grafted onto naïve, syngeneic, wild type mice was not spontaneously rejected in spite of an E7-specific humoral and cellular immune response promoted in graft recipients (11, 12). Furthermore, immunisation with vaccines that are known to reject E7 producing transplantable tumours, also failed to reject E7 skin grafts (11, 12). This result contrasts to similar skin-grafting experiments

where other proteins are used as transgenes, e.g, ovalbumin protein (OVA) & human growth hormone: such grafts spontaneously reject within 20 days (13, 14). These findings suggested that the fate of grafts is antigen dependant, and suggested the existence of a mechanism leading to E7 tolerance which could explain the failure of E7 therapeutic vaccination.

Materials and Methods

Mice. Specific pathogen-free FVB/N (H-2^q) mice were obtained from the Animal Resources Centre (Perth, Australia). C57BL/6 (H-2^b) wild-type mice, C57BL/6 and FVB/N mice transgenic for the HPV16 E7 oncoprotein driven from a K14 promoter were obtained from the Princess Alexandra Hospital Biological Resources Facility (Brisbane, Australia), where HPV16 E7 transgenic C57BL/6 mice were backcrossed with FVB/N mice to generate (K14E7.C57BL/6 x FVB/N) F1 mice. Description of these mice and of the transgene expression has been previously reported (10, 11). All mice were females between 6 and 12 weeks of age, and were maintained under specific pathogen-free conditions at the Princess Alexandra Hospital Biological Resources Facility.

Skin Grafting. An allograft [FVB/N x C57BL/6 (H-2^{qxb})] expressing E7 from a keratin 14 promoter, or not expressing E7, was grafted adjacent to an autograft expressing E7 on FVB/N (H-2^q) mice (Fig 1). Groups of 4 mice were grafted at a time, and the experiment was repeated two more times. The results shown are pooled from the three experiments. Whole-thickness ear skin grafting was performed as previously described (12). Grafts vascularised and completely adherent seven days post-surgery were judged technically successful. Grafts were observed at least twice weekly for the duration of experiments. Grafts intact past 20 days after grafting were

considered healed. Grafts intact at day 200 post-surgery were retained as non-rejected. Skin graft rejection was assessed as described (14).

$CD4^+$ and $CD8^+$ T Cell Depletion. $CD4^+$ T cells were depleted using anti-CD4 mAb (clone GK1.5) by i.p injections of 1.0 mg/mouse for each of three injections, two days apart. The first injection of Ab was given either 10 days before grafting, or 15 days after grafting. $CD8^+$ T cells were depleted using 200μg/mouse of anti-CD8 mAb (clone 53-5.8) by i.p. injections at day 14 and day 2 before grafting. Depletion was checked using flow cytometry as previously described (15). More than 95% of the cell population of interest was shown to be depleted (data not shown).

Data Analysis. All graphs were generated using GraphPad Prism version 5.01 (La Jolla, CA 92037, USA), with the statistics calculated by using the Log rank test with a 95% confidence interval. Results were considered significant where the P value was ≤ 0.05.

Results and Discussion

Allo priming allows rejection of the HPV 16 E7 oncoprotein-producing syngeneic skin graft which is resistant to vaccination.

MHC matched skin grafts expressing the HPV 16 E7 protein (E7 autograft) are not spontaneously rejected from an immunocompetent host (Fig 2A). However simultaneous placement of an allograft next to an E7 autograft results in the E7 autograft rejection. The allografts were rejected in an average of 14 days or less as expected (data not shown), and induce an immune response which allows for the rejection of the E7-autograft in an average of 104 days (Fig. 2A).

CD8$^+$ T cells are essential for E7 autograft rejection.

When CD8$^+$ cells are depleted before grafting, none of the E7 autografts are rejected (Fig. 2B), indicating that the presence of CD8$^+$ T cells is essential during priming for the E7 autograft rejection process. A follow-up of E7 autografts up to 200 days after grafting showed that there was no rejection. By this time the CD8$^+$ population had recovered (16). Therefore the presence of unprimed CD8$^+$ T cells is not sufficient for the rejection process.

CD4$^+$ T cells depletion hastens E7 autograft rejection.

Next we determined the role of CD4$^+$ T cells in the E7 autograft rejection. CD4$^+$ T cells were depleted before grafting or 15 days after grafting. We wished to determine whether CD4$^+$ T cells are directly involved in the graft rejection or providing help indirectly to CD8$^+$ T cells to convert them to efficient killer cells. In contrast to our expectations, when the CD4$^+$ T cells were depleted before grafting, the autograft rejection is accelerated compared to non-depleted control (average time of rejection 44 days vs 104 days after grafting, Fig 2C). CD4$^+$ T cells are therefore slowing the E7 autograft rejection. We hypothesize that a subtype of CD4$^+$ T cell population (probably regulatory T cells or Treg) is suppressing the immune response driven by antigen specific CD8$^+$ T cells. Others have observed that there are more CD4+Foxp3+ Treg cells in the E7 expressing skin compared with control mouse skin (17, and personal communication). Depleting CD4$^+$ T cells may have impaired this suppressor function, and therefore amplified the response leading to graft rejection. A recent clinical study in which a synthetic long E7 and E6 peptide was used as a vaccine for women with cervical cancer showed that vaccinated women exhibited expanded levels of CD4$^+$CD25$^+$Foxp3$^+$ Treg cell population (18). These findings

agree with our data and suggest E7 inhibits induction of immunity to itself through a $CD4^+$ (possibly $CD25^+$ regulatory) T cell dependant mechanism.

If $CD4^+$ T cells are depleted after grafting, again the graft rejection process is hastened compared to the untreated mice. However the average time needed to reject the E7 autograft is slightly longer than when the $CD4^+$ T cells are depleted before grafting (52.6 days vs 44 days, Fig 2C). This may indicate that shorter duration of $CD4^+$ T cell in contact with the E7 antigen in this case have induced lower amounts of suppressor T cells. In the presence of the graft, $CD4^+$ T cells may start to suppress the $CD8^+$ T cell response. Once $CD4^+$ T cells are depleted, the suppressive signal to $CD8^+$ T cells may be switched off and the rejection process can take place. Another possible explanation is that some other lymphocyte populations such as $CD8^+$ T cells or NK T cells expanded to fill in the vacuum created by $CD4^+$ T cell depletion, and rejected the autografts more efficiently. Twenty days after grafting, we stopped anti-CD4 mAb injections, allowing $CD4^+$ populations (including Tregs) to recover. It has been shown that more than 50% of the entire $CD4^+$ T cell population depleted using our antibody regimen recovered 55 days post injection (16). This may explain why after 61 days, no more E7 autograft rejection is observed.

Absence of E7 in the allograft promotes the fastest rejection rate for E7 Autografts.

When the E7-autograft is grafted simultaneously with an allograft that does not produce E7, the rejection of the E7 autograft is quicker than observed with any of the other experiments (average time of rejection=27 days compared with the average of 104 days when the allograft is producing E7) (Fig 2D). This observation indicates that the E7 protein in the allograft is also actively suppressing E7 autograft rejection by possibly promoting the generation of $CD4^+$ Treg cells that will slow down the

rejection process of the E7 autograft. This correlates well with our data on $CD4^+$ T cell depleted animals. It is interesting to note that the depletion of $CD4^+$ T cells either before or after grafting, hastened the mean time of rejection of the E7-autograft to about 40 days, similar to what is observed with the E7 negative allograft induced response. Thus we conclude that the induction of specific immunity to E7 was inhibited by the expression of E7 in the allograft, and the inhibition mechanism potentially involves $CD4^+$ T cells. Therefore the efficacy of allo priming as a means to induce tumour-specific immunity may depend on whether a simultaneous regulatory $CD4^+$ T cells population was induced or not.

Acknowledgements

This study was performed as part of the honours thesis (Marion Brunck) and was supported by a grant (ID# 301084) from the National Health and Medical Research Council of Australia. We thank Professor Ian Frazer, Dr. Graham Leggatt and Dr. Rachel De Kluyver for helpful discussions. We also thank the animal house staff Ms. Kerry Fagan, Ms. Caron Maxim, and Ms. Megan Bathurst for excellent animal care and Thomas Brunck for his help with the design of Figure 1.

Legends

Fig. 1. Mouse Model

A mouse model for cervical cancer was used in which E7-expressing skin (K14E7.FVB/N) is grafted onto wild-type FVB/N syngeneic recipient mice. Rejection of this E7-autograft was induced by the simultaneous co-grafting adjacent to the E7 autograft, of an MHC-mismatched skin (K14E7.C57BL/6 x FVB/N)F1. We selectively depleted $CD8^+$ (14 days before grafting) or $CD4^+$ T cells (10 days before grafting or 15 days after grafting) to investigate the role of those T cell populations in the rejection of the E7-autograft.

Fig. 2.

Survival of E7 skin autografts on FVB/N mice.

2A. FVB/N mice were grafted with E7.FVB/N skin (E7 Autograft) only (Δ) or simultaneously co-grafted with (E7.C57BL/6 x FVB/N)F1 skin (E7 Allograft) (●). Kaplan-Meier analysis of survival of the E7 autograft was performed. There was no rejection observed even after 200 days post-surgery in the group grafted only with E7-autograft. 5 out of 12 mice from the double-grafted groups rejected the E7 autograft between day 90 and 125 post-surgery (Average 104 days) (statistically significant: Mantel-Cox test, p=0.013).

2B. FVB/N mice were simultaneously co-grafted with E7.FVB/N skin (E7 Autograft) and (E7.C57BL/6 x FVB/N)F1 skin (E7 Allograft). Graft recipients were untreated (●), or treated with anti-CD8 mAb (clone 53-5.8) day 14 and day 2 before grafting (Δ). None of the $CD8^+$ T cell depleted mice rejected the E7-autograft even 200 days post-surgery (p=0.013).

2C. FVB/N mice were simultaneously co-grafted with E7.FVB/N skin (E7 Autograft) and (E7.C57BL/6 x FVB/N)F1 skin (E7 Allograft). Mice were untreated (●), or treated with anti-CD4 mAb (clone GK1.5) either 10, 8 and 6 days before grafting (Δ), or 15, 17 and 19 days after grafting (▼). Survival of E7 grafts of non-depleted control versus group depleted before grafting: p=0.36; survival of E7 grafts of non-depleted control versus group depleted after grafting: p=0.51; survival of E7 grafts of group depleted before grafting versus group depleted after grafting: p=0.11.

2D. FVB/N mice were simultaneously co-grafted with E7.FVB/N skin (E7 Autograft) and either E7 positive or E7 negative (C57BL/6 x FVB/N)F1 allograft skin. Graft survival percentage is shown for the E7 autografts when co-grafted with E7 negative allografts (Δ) or E7 positive allografts (●). Median survival day = 27 (E7 negative group) vs 103 (E7 positive control), p=0.013.

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Figures

Fig. 1.

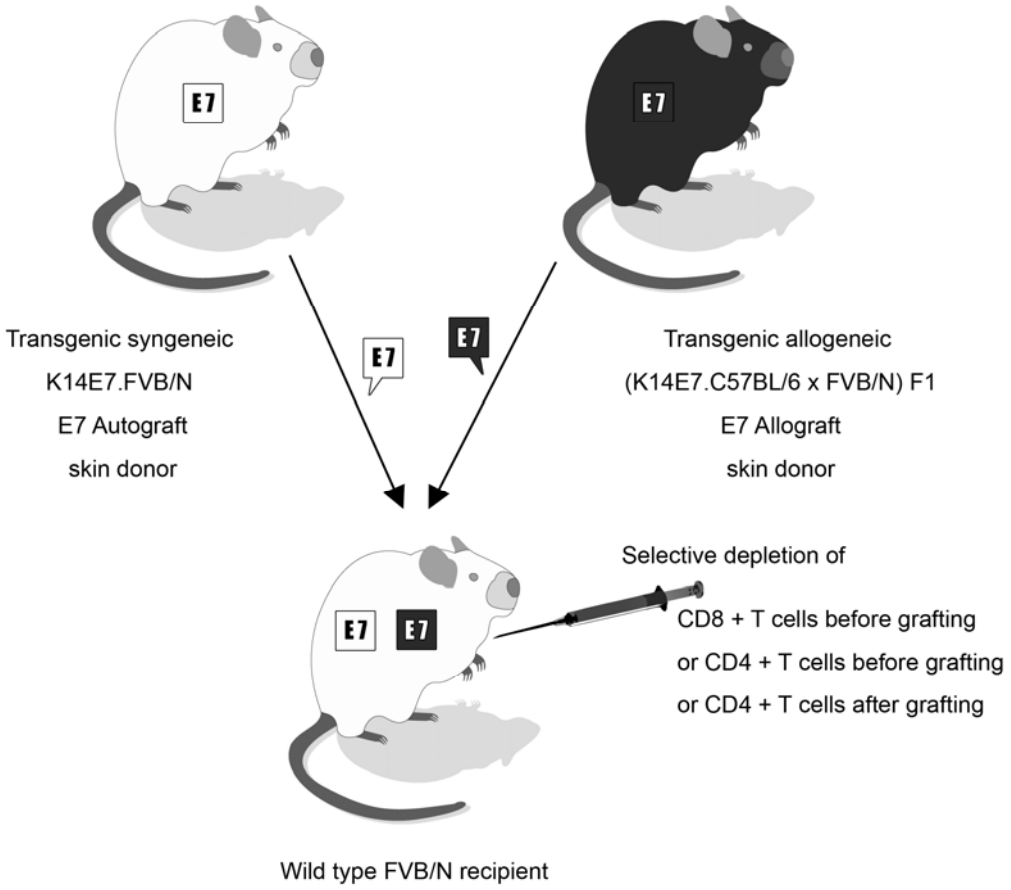

Fig. 2.